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Mullis et al.

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[54] **PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES**

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[*] Notice: The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed.

[21] Appl. No.: **828,144**

[22] Filed: **Feb. 7, 1986**

Related U.S. Application Data

[60] Continuation-in-part of Ser. No. 824,044, Jan. 30, 1986, abandoned, which is a division of Ser. No. 791,308, Oct. 25, 1985, which is a continuation-in-part of Ser. No. 716,975, Mar. 28, 1985, abandoned.

[51] **Int. Cl.**[4] **C12Q 1/68;** C12P 19/34; C12N 1/00; C12N 15/00; G01N 33/48; G01N 33/00; G01N 33/566; G01N 33/564; C07H 21/02; C07H 21/04

[52] **U.S. Cl.** **435/6;** 435/91; 435/172.3; 435/317; 436/63; 436/94; 436/501; 436/508; 536/27; 536/28; 536/29; 935/17; 935/18; 935/76; 935/77; 935/78

[58] **Field of Search** 435/91, 172.3, 317, 435/6; 536/27, 28, 29; 935/17, 18, 78, 77, 76; 436/63, 94, 501, 508

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[57] **ABSTRACT**

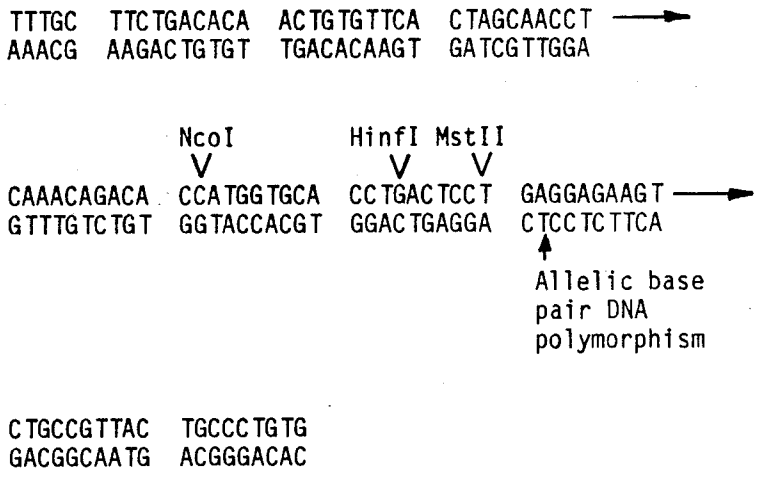
The present invention is directed to a process for amplifying and detecting any target nucleic acid sequence contained in a nucleic acid or mixture thereof. The process comprises treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers, extending the primers to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence, and detecting the sequence so amplified. The steps of the reaction may be carried out stepwise or simultaneously and can be repeated as often as desired.

In addition, a specific nucleic acid sequence may be cloned into a vector by using primers to amplify the sequence, which contain restriction sites on their non-complementary ends, and a nucleic acid fragment may be prepared from an existing shorter fragment using the amplification process.

26 Claims, 12 Drawing Figures

FIG.1

Double-Stranded 94-bp Sequence



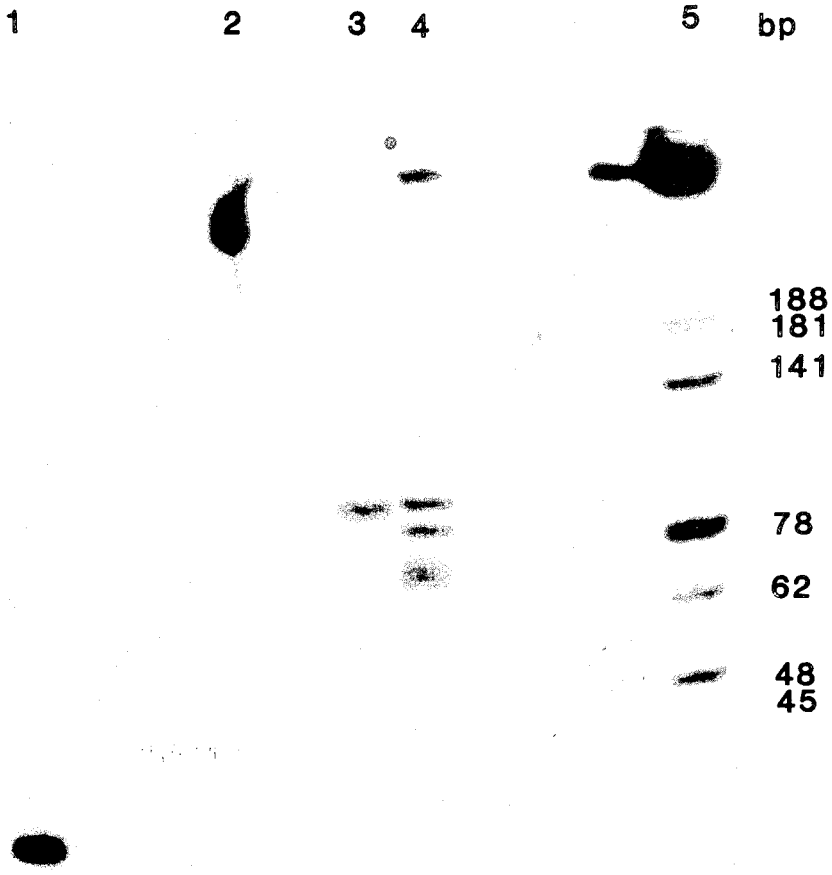


FIG.2

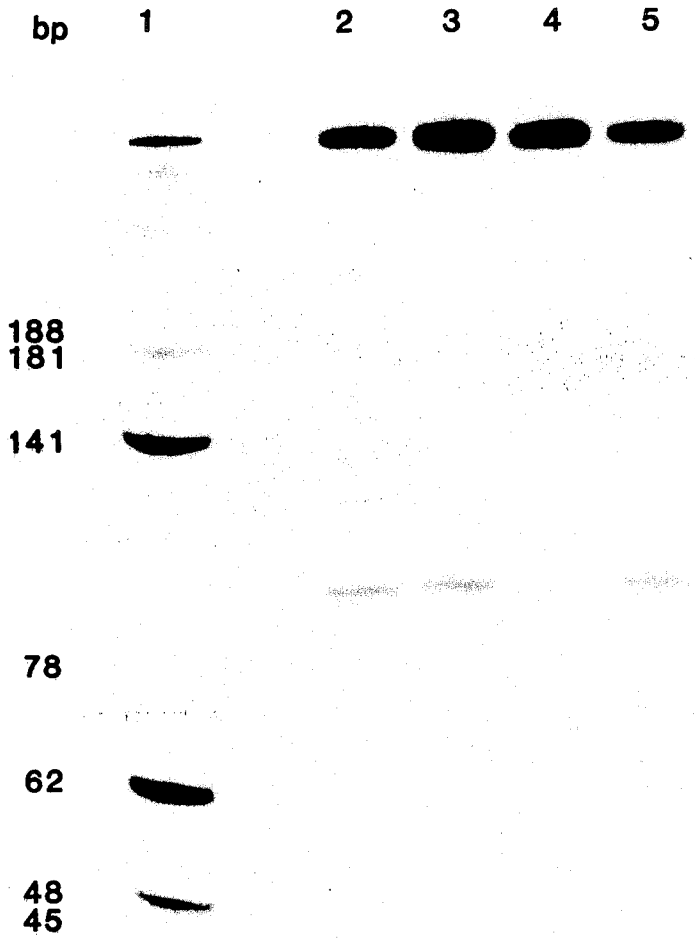


FIG.3

FIG.4-1

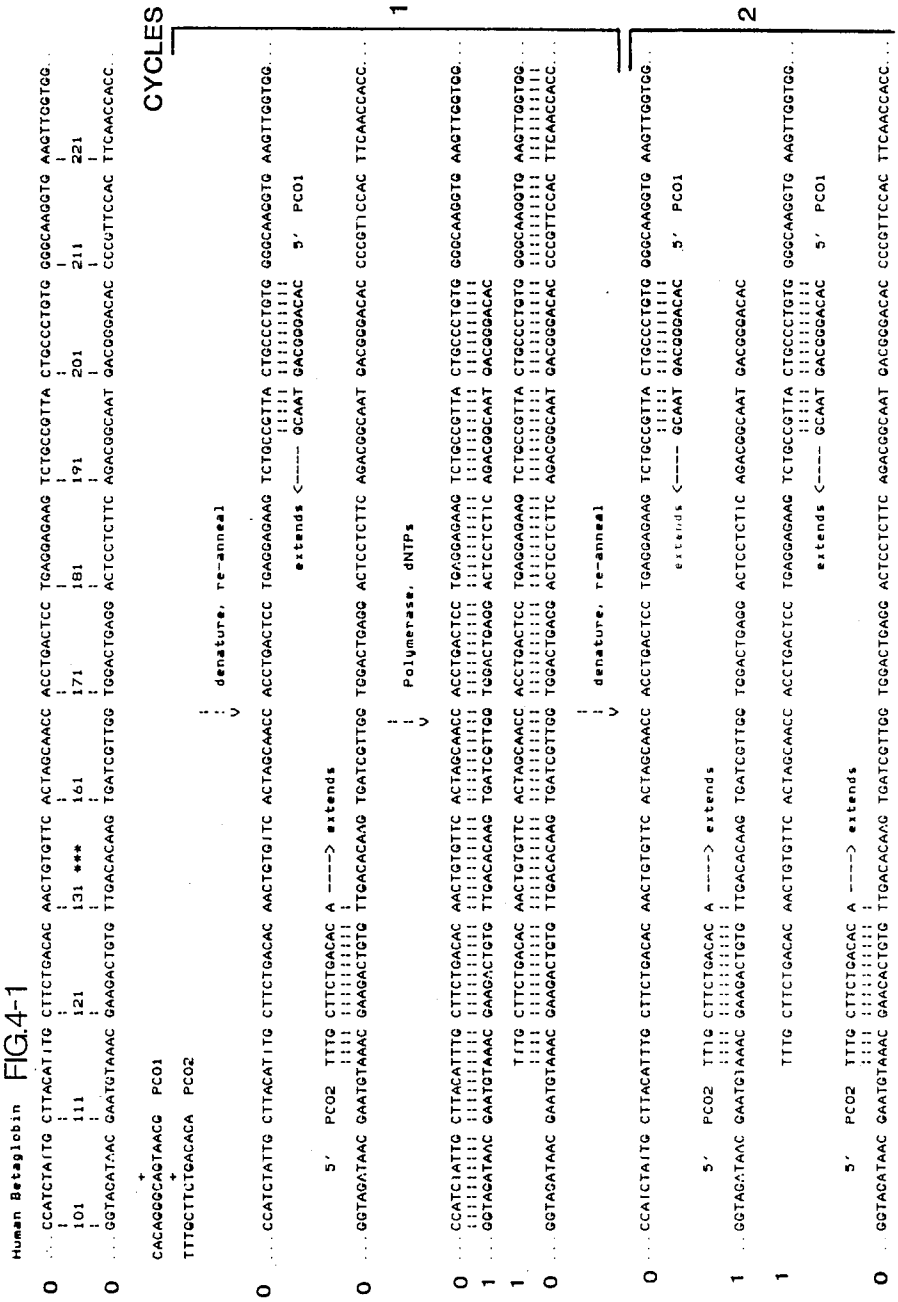

Human Betaglobin
CYCLES
PCO1
PCO2
denature, re-anneal
Polymerase, dNTPs
extends
5'

FIG.4-2

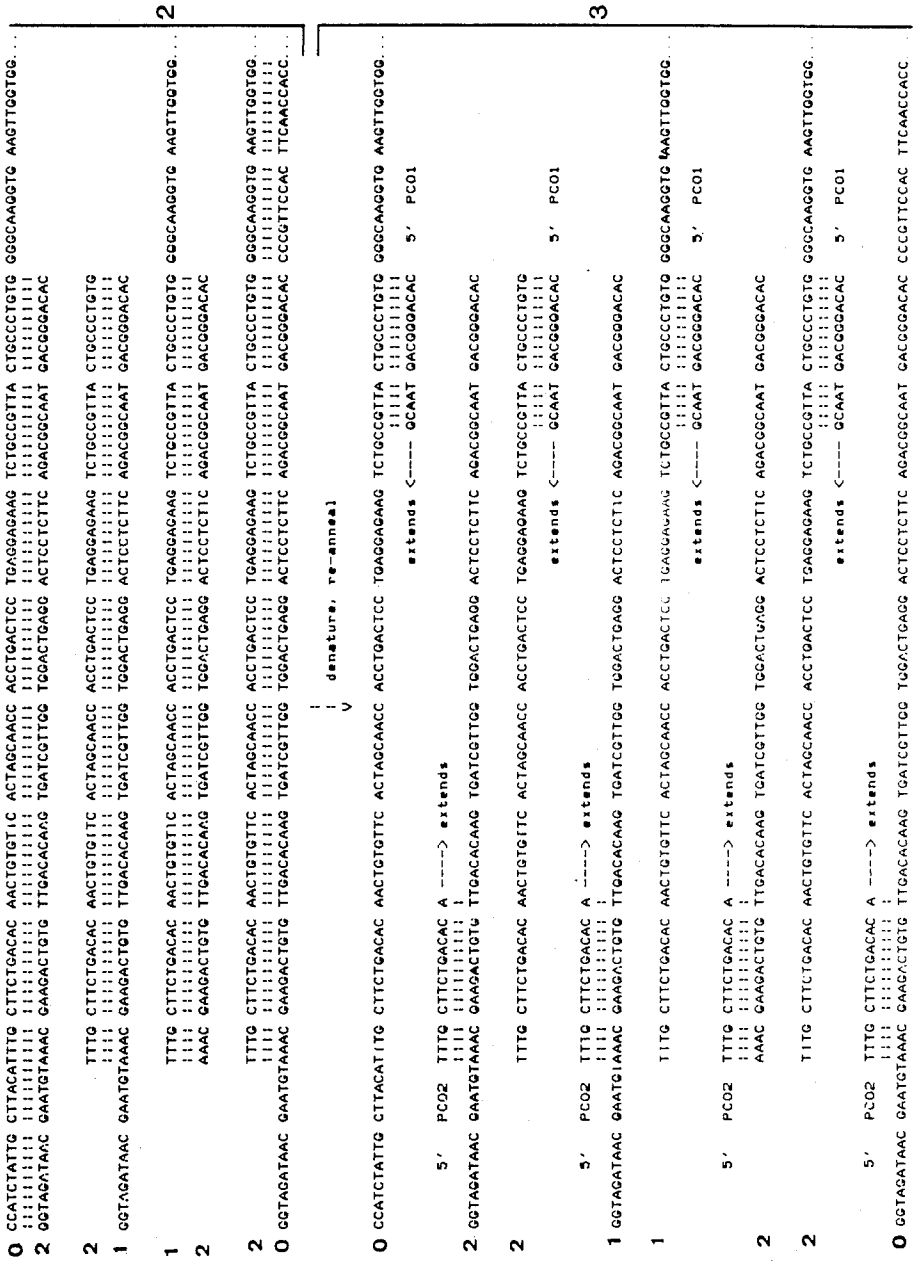
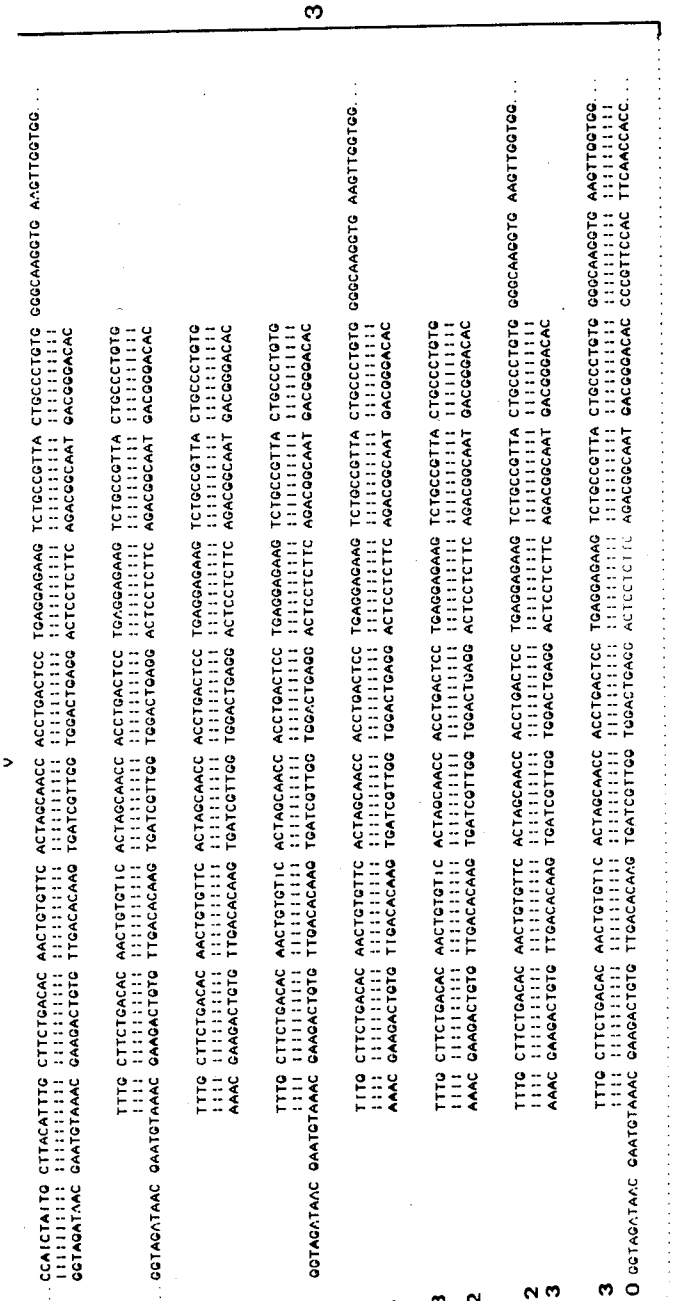

Polymerase, dNTPs
denature, re-anneal
extends
5' PCO1
5' PCO2
2
3

FIG.4-3


Polymerase, dNTPs
COPIES OF DNA SEQUENCE AFTER N CYCLES
N
template
long product
short product
=2(exp N)-N-1

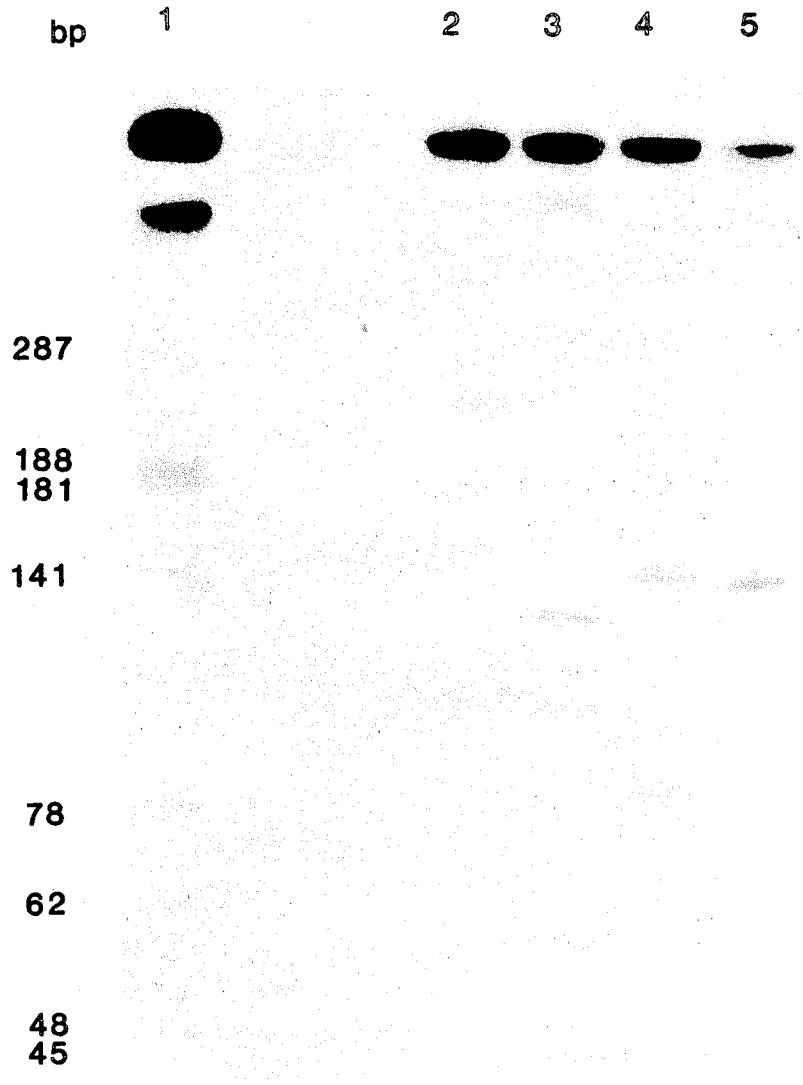
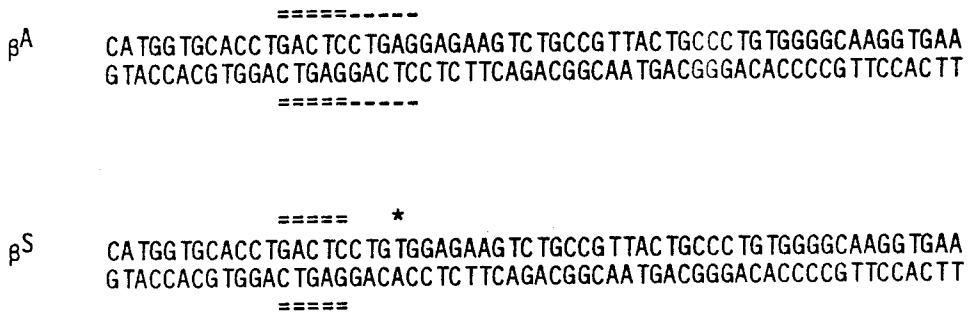


FIG.5

FIG.6



* Marks the mutation (A to T) in the sickle cell gene which disrupts the DdeI site

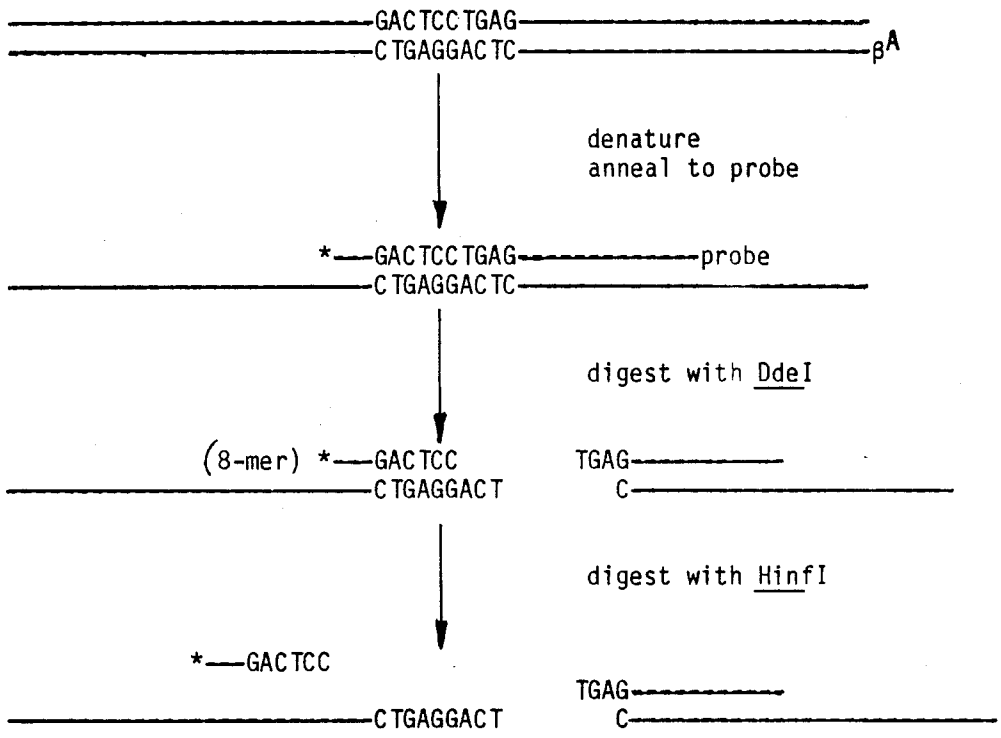


FIG.7

* is label

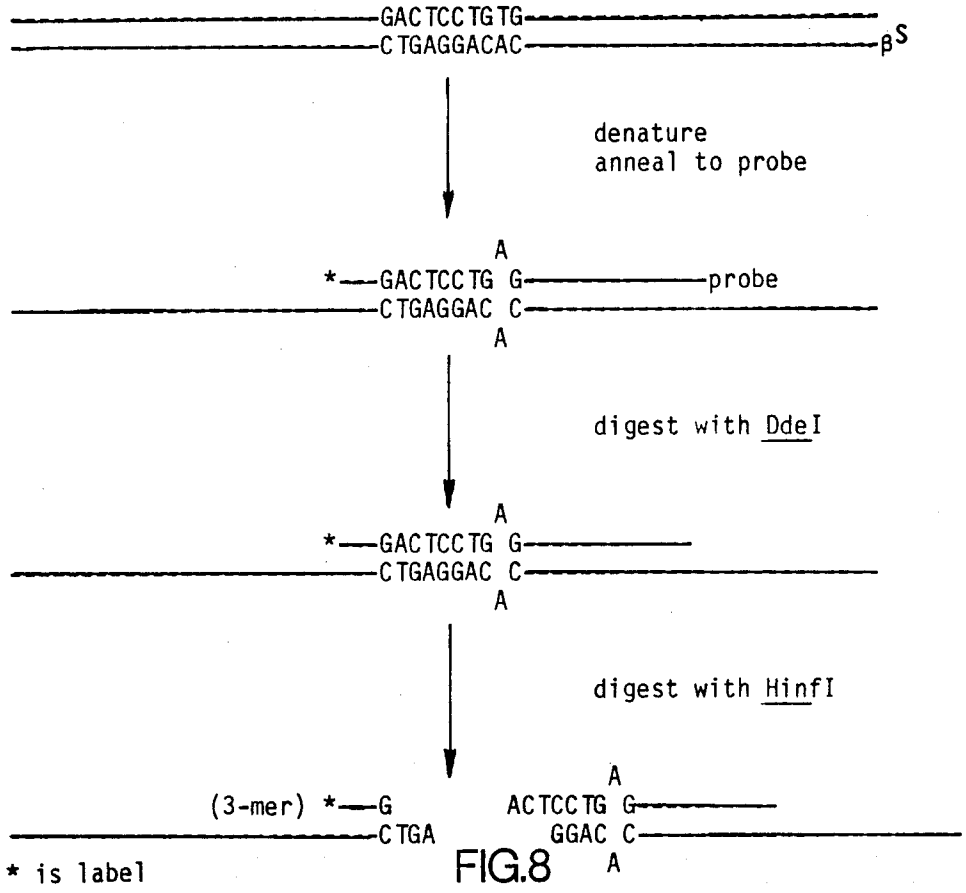

GACTCCTGTG
CTGAGGACAC
βS
denature
anneal to probe
A
*—GACTCCTG G
probe
CTGAGGAC C
A
digest with DdeI
A
*—GACTCCTG G
CTGAGGAC C
A
digest with HinfI
A
(3-mer) *—G
ACTCCTG G
CTGA
GGAC C
A
* is label

FIG.8

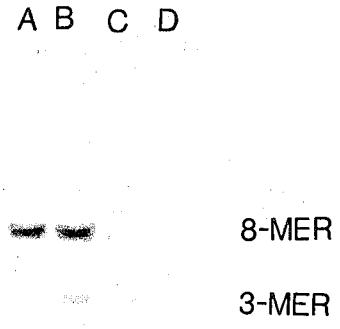
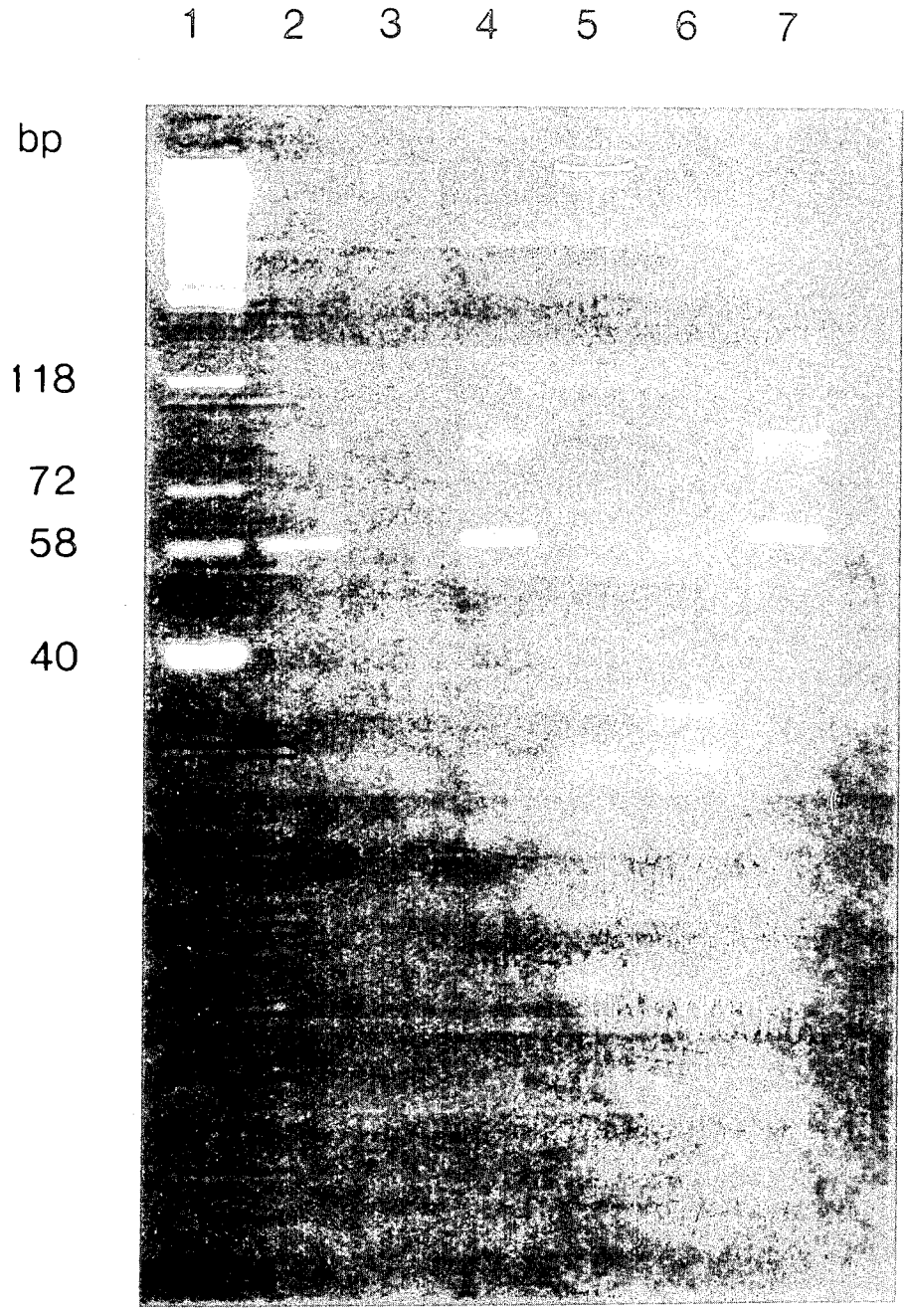


FIG.9

FIG. 10



PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES

BACKGROUND OF THE INVENTION

This application is a continuation-in-part application of copending U.S. Ser. No. 824,044 filed Jan. 30, 1986, now abandoned, which is a divisional application of copending U.S. Ser. No. 791,308 filed Oct. 25, 1985, which is a continuation-in-part application of copending U.S. application Ser. No. 716,975 filed Mar. 28, 1985, now abandoned.

FIELD OF THE INVENTION

The present invention relates to a process for amplifying existing nucleic acid sequences if they are present in a test sample and detecting them if present by using a probe. More specifically, it relates to a process for producing any particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present so as to facilitate detection of the sequences. The DNA or RNA may be single- or double-stranded, and may be a relatively pure species or a component of a mixture of nucleic acids. The process of the invention utilizes a repetitive reaction to accomplish the amplification of the desired nucleic acid sequence.

DESCRIPTION OF RELATED DISCLOSURES

For diagnostic applications in particular, the target nucleic acid sequence may be only a small portion of the DNA or RNA in question, so that it may be difficult to detect its presence using nonisotopically labeled or end-labeled oligonucleotide probes. Much effort is being expended in increasing the sensitivity of the probe detection systems, but little research has been conducted on amplifying the target sequence so that it is present in quantities sufficient to be readily detectable using currently available methods.

Several methods have been described in the literature for the synthesis of nucleic acids de novo or from an existing sequence. These methods are capable of producing large amounts of a given nucleic acid of completely specified sequence.

One known method for synthesizing nucleic acids de novo involves the organic synthesis of a nucleic acid from nucleoside derivatives. This synthesis may be performed in solution or on a solid support. One type of organic synthesis is the phosphotriester method, which has been utilized to prepare gene fragments or short genes. In the phosphotriester method, oligonucleotides are prepared which can then be joined together to form longer nucleic acids. For a description of this method, see Narang, S. A., et al., *Meth. Enzymol.*, 68, 90 (1979) and U.S. Pat. No. 4,356,270. The patent describes the synthesis and cloning of the somatostatin gene.

A second type of organic synthesis is the phosphodiester method, which has been utilized to prepare a tRNA gene. See Brown, E. L., et al., *Meth. Enzymol.*, 68, 109 (1979) for a description of this method. As in the phosphotriester method, the phosphodiester method involves synthesis of oligonucleotides which are subsequently joined together to form the desired nucleic acid.

Although the above processes for de novo synthesis may be utilized to synthesize long strands of nucleic acid, they are not very practical to use for the synthesis of large amounts of a nucleic acid. Both processes are laborious and time-consuming, require expensive equipment and reagents, and have a low overall efficiency. The low overall efficiency may be caused by the inefficiencies of the synthesis of the oligonucleotides and of the joining reactions. In the synthesis of a long nucleic acid, or even in the synthesis of a large amount of a shorter nucleic acid, many oligonucleotides would need to be synthesized and many joining reactions would be required. Consequently, these methods would not be practical for synthesizing large amounts of any desired nucleic acid.

Methods also exist for producing nucleic acids in large amounts from small amounts of the initial existing nucleic acid. These methods involve the cloning of a nucleic acid in the appropriate host system, where the desired nucleic acid is inserted into an appropriate vector which is used to transform the host. When the host is cultured the vector is replicated, and hence more copies of the desired nucleic acid are produced. For a brief description of subcloning nucleic acid fragments, see Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, pp. 390–401 (1982). See also the techniques described in U.S. Pat. Nos. 4,416,988 and 4,403,036.

A third method for synthesizing nucleic acids, described in U.S. Pat. No. 4,293,652, is a hybrid of the above-described organic synthesis and molecular cloning methods. In this process, the appropriate number of oligonucleotides to make up the desired nucleic acid sequence is organically synthesized and inserted sequentially into a vector which is amplified by growth prior to each succeeding insertion.

The present invention bears some similarity to the molecular cloning method; however, it does not involve the propagation of any organism and thereby avoids the possible hazards or inconvenience which this entails. The present invention also does not require synthesis of nucleic acid sequences unrelated to the desired sequence, and thereby the present invention obviates the need for extensive purification of the product from a complicated biological mixture.

SUMMARY OF THE INVENTION

The present invention resides in a process for amplifying one or more specific nucleic acid sequences present in a nucleic acid or mixture thereof using primers and agents for polymerization and then detecting the amplified sequence. The extension product of one primer when hybridized to the other becomes a template for the production of the desired specific nucleic acid sequence, and vice versa, and the process is repeated as often as is necessary to produce the desired amount of the sequence. This method is expected to be more efficient than the methods described above for producing large amounts of nucleic acid from a target sequence and to produce such nucleic acid in a comparatively short period of time. The present method is especially useful for amplifying rare species of nucleic acid present in a mixture of nucleic acids for effective detection of such species.

More specifically, the present invention provides a process for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different forms of sequences in said sample, wherein the sample is suspected of contain-

ing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence suspected of being present in the sample, under hybridizing conditions such that for each strand of each different sequence to be detected an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be substantially complementary to each strand of each specific sequence such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present;

(c) treating the sample with oligonucleotides primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the specific nucleic acid sequence or sequences if present;

(d) adding to the product of step (c) a labeled probe capable of hybridizing to said sequence being detected or a mutation thereof; and

(e) determining whether said hybridization has occurred.

The steps (a)–(c) may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence amplification is obtained.

In other embodiments the invention relates to diagnostic kits for the detection of at least one specific nucleic acid sequence in a sample containing one or more nucleic acids at least one of which nucleic acid is suspected of containing said sequence, which kit comprises, in packaged form, a multicontainer unit having

(a) one container for each oligonucleotide primer for each strand of each different sequence to be detected, which primer or primers are substantially complementary to each strand of each specific nucleic acid sequence such that an extension product synthesized from one primer, when it is separated from its complement, can serve as a template for the synthesis of the extension product of the other primer;

(b) a container containing an agent for polymerization;

(c) a container for each of four different nucleoside triphosphates;

(d) a container containing a probe capable of detecting the presence of said sequence in said sample; and

(e) a container containing means for detecting hybrids of said probe and said sequence.

In yet another embodiment, the invention relates to a process for cloning into a vector a specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, which process comprises:

(a) treating the nucleic acid(s) with one oligonucleotide primer for each strand of each different specific sequence being amplified, under conditions such that for each strand of each different sequence being amplified an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be substantially complementary to each strand of each specific sequence such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein said primer or primers each contain a restriction site on its 5′ end which is the same as or different from the restriction site(s) on the other primer(s);

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, wherein depending on the particular sequence being amplified, steps (a) and (c) are carried out in the presence of from 0 up to an effective amount of dimethylsulfoxide or at a temperature of up to about 45° C.;

(d) adding to the product of step (c) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(e) ligating the cleaved product(s) into one or more cloning vectors.

In yet another embodiment, the invention herein relates to a process for synthesizing a nucleic acid fragment from an existing nucleic acid fragment having fewer nucleotides than the fragment being synthesized and two oligonucleotide primers, wherein the nucleic acid being synthesized is comprised of a left segment, a core segment and a right segment, and wherein the core segment represents at least substantially the nucleotide sequence of said existing nucleic acid fragment, and the right and left segments represent the sequence nucleotide present in the 5′ ends of the two primers, the 3′ ends of which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of said existing nucleic acid fragment, which process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under condition such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to the 3′ end of each strand of said existing fragment such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the product of step (d).

The core fragment may be obtained by the steps comprising:

(a) reacting two oligonucleotides, each of which contain at their 3′ ends a nucleotide sequence which is complementary to the other oligonucleotide at its 3′ end, and which are non-complementary to each other at their 5′ ends, with an agent for polymerization and four nucleoside triphosphates under conditions such that an extension product of each oligonucleotide is synthesized which is complementary to each nucleic acid strand;

(b) separating the extension products from the templates on which they were synthesized to produce single-stranded molecules; and

(c) treating the single-stranded molecules generated from step (b) with the oligonucleotides of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the core fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates a 94 base pair length sequence of human β-globin desired to be amplified. The single base pair change which is associated with sickle cell anemia is depicted beneath the 94-mer.

FIG. **2** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of the 94-mer contained in human wild-type DNA and in a plasmid containing a 1.9 kb BamHI fragment of the normal β-globin gene (pBR328:HbA).

FIG. **3** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of any of the specific target 94-mer sequence present in pBR328:HbA, a plasmid containing a 1.9 kb BamHI fragment of the sickle cell allele of β-globin (pBR328:HbS), pBR328:HbA where the sequence to be amplified is cleaved with MstII, and pBR328:HbS where the sequence to be amplified has been treated but not cleaved with MstII.

FIG. **4** illustrates in detail the steps and products of the polymerase chain reaction for amplification of the desired 94-mer sequence of human β-globin for three cycles using two oligonucleotides primers.

FIG. **5** represents a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification after four cycles of a 240-mer sequence in pBR328:HbA, where the aliquots are digested with NcoI (Lane 3), MstII (Lane 4) or HinfI (Lane 5). Lane 1 is the molecular weight standard and Lane 2 contains the intact 240-bp product.

FIG. **6** illustrates the sequence of the normal (β^A) and sickle cell (β^S) β-globin genes in the region of the DdeI and HinfI restriction sites, where the single lines for β^A mark the position of the DdeI site (CTGAG) and the double bars for β^A and β^S mark the position of the HinfI site (GACTC).

FIG. **7** illustrates the results of sequential digestion of normal β-globin using a 40-mer probe and DdeI followed by HinfI restriction enzymes.

FIG. **8** illustrates the results of sequential digestion of sickle β-globin using the same 40-mer probe as in FIG. **7** and DdeI followed by HinfI restriction enzymes.

FIG. **9** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating the use of the same 40-mer probe as in FIG. **7** to specifically characterize the beta-globin alleles present in samples of whole human DNA which have been subjected to amplification, hybridization with the probe, and sequential digestion with DdeI and HinfI.

FIG. **10** illustrates a photograph of a 6% NuSieve agarose gel visualized using ethidium bromide and UV light. This photograph demonstrates amplification of a sub-fragment of a 110-bp amplification product which sub-fragment is an inner nested set within the 110-bp fragment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein in referring to primers, probes, oligomer fragments to be detected, oligomer controls and unlabeled blocking oligomers is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15–25 or more nucleotides, although it may contain fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5′ end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be am-

plified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism" refers to the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

The term "restriction fragment length polymorphism" ("RFLP") refers to the differences in DNA nucleotide sequences that are randomly distributed throughout the entire human genome and that produce different restriction endonuclease patterns.

The present invention is directed to a process for amplifying any one or more desired specific nucleic acid sequences suspected of being in a nucleic acid. Because large amounts of a specific sequence may be produced by this process, the present invention may be used for improving the efficiency of cloning DNA or messenger RNA and for amplifying a target sequence to facilitate detection thereof.

In general, the present process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given (a) that the ends of the required sequence are known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and (b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any source of nucleic acid, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it is suspected of containing the specific nucleic acid sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single stranded or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA or a portion of nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi or amniotic cells by a variety of techniques such as that described by Maniatis et al., *Molecular Cloning: A Laboratory Manual,* (New York: Cold Spring Harbor Laboratory, 1982), pp 280-281.

Any specific nucleic acid sequence can be produced by the present process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid of defined length. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the efficiency of the process. It will be understood that the word primer as used hereinafter may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified. For instance, in the case where a nucleic acid sequence is inferred from protein sequence information a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the desired sequence to be amplified.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* (1981), 22:1859-1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template, either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation may involve temperature ranging from about 80° to 105° C. for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by *Cold Spring Harbor Symposia on Quantitative Biology,* Vol. XLIII "DNA: Replication and Recombination" (New York:

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Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63–67, and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics,* 16:405–37 (1982).

If the original nucleic acid containing the sequence to be amplified is single stranded, its complement is synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, an agent for polymerization and the four nucleotides described below. The product will be partially complementary to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of unequal length strands that may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added to the single-stranded nucleic acid and the reaction carried out.

If the original nucleic acid constitutes the sequence to be amplified, the primer extension product(s) produced will be completely complementary to the strands of the original nucleic acid and will hybridize therewith to form a duplex of equal length strands to be separated into single-stranded molecules.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7–9, most preferably about 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6: 1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP and TTP are also added to the synthesis mixture in adequate amounts and the resulting solution is heated to about 90°–100° C. for from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to from 20°–40° C., which is preferable for the primer hybridization. To the cooled mixture is added an agent for polymerization, and the reaction is allowed to occur under conditions known in the art. This synthesis reaction may occur at from room temperature up to a temperature above which the agent for polymerization no longer functions efficiently. Thus, for example, if DNA polymerase is used as the agent for polymerization, the temperature is generally no greater than about 45° C. Preferably an amount of dimethylsulfoxide (DMSO) is present which is effective in detection of the signal or the temperature is 35°–40° C. Most preferably, 5–10% by volume DMSO is present and the temperature is 35°–40° C. For certain applications, where the sequences to be amplified are over 110 base pair fragments, such as the HLA DQ-α or -β genes, an effective amount (e.g., 10% by volume) of DMSO is added to the amplification mixture, and the reaction is carried at 35°–40° C., to obtain detectable results or to enable cloning.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heatstable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3′ end of each primer and proceed in the 5′ direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents, however, which initiate synthesis at the 5′ end and proceed in the other direction, using the same process as described above.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional inducing agent, nucleotides and primers may be added if necessary for the reaction to proceed under the conditions prescribed above. Again, the synthesis will be initiated at one end of the oligonucleotide primers and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleic acid sequence bounded by the two primers.

The steps of strand separation and extension product synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence. As will be described in further detail below, the amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

When it is desired to produce more than one specific nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

The present invention can be peformed in a step-wise fashion where after each step new reagents are added, or simultaneously, where all reagents are added at the initial step, or partially step-wise and partially simultaneous, where fresh reagent is added after a given number of steps. If a method of strand separation, such as heat, is employed which will inactivate the agent for polymerization, as in the case of a heat-labile enzyme, then it is necessary to replenish the agent for polymerization after every strand separation step. The simultaneous method may be utilized when a number of puri-

fied components, including an enzymatic means such as helicase, is used for the strand separation step. In the simultaneous procedure, the reaction mixture may contain, in addition to the nucleic acid strand(s) containing the desired sequence, the strand-separating enzyme (e.g., helicase), an appropriate energy source for the strand-separating enzyme, such as rATP, the four nucleotides, the oligonucleotide primers in molar excess, and the inducing agent, e.g., Klenow fragment of *E. coli* DNA polymerase I. If heat is used for denaturation in a simultaneous process, a heat-stable inducing agent such as a thermostable polymerase may be employed which will operate at an elevated temperature, preferably 65°-90° C. depending on the inducing agent, at which temperature the nucleic acid will consist of single and double strands in equilibrium. For smaller lengths of nucleic acid, lower temperatures of about 50° C. may be employed. The upper temperature will depend on the temperature at which the enzyme will degrade or the temperature above which an insufficient level of primer hybridization will occur. Such a heat-stable enzyme is described, e.g., by A. S. Kaledin et al., *Biokhimiya*, 45, 644–651 (1980). Each step of the process will occur sequentially notwithstanding the initial presence of all the reagents. Additional materials may be added as necessary. After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzymes in any known manner or separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an automated process, the reaction may be cycled through a denaturing region, a reagent addition region, and a reaction region. In another embodiment, the enzyme used for the synthesis of primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series; thus the nucleic acids produced can be repeatedly denatured without inactivating the enzyme.

The present invention is demonstrated diagrammatically below where double-stranded DNA containing the desired sequence [S] comprised of complementary strands [S+] and [S−] is utilized as the nucleic acid. During the first and each subsequent reaction cycle extension of each oligonucleotide primer on the original template will produce one new ssDNA molecule product of indefinite length which terminates with only one of the primers. These products, hereafter referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleotide primers during subsequent cycles and will produce molecules of the desired sequence [S+] or [S−]. These molecules will also function as templates for one or the other of the oligonucleotide primers, producing further [S+] and [S−], and thus a chain reaction can be sustained which will result in the accumulation of [S] at an exponential rate relative to the number of cycles.

By-products formed by oligonucleotide hybridizations other than those intended are not self-catalytic (except in rare instances) and thus accumulate at a linear rate.

The specific sequence to be amplified, [S], can be depicted diagrammatically as:

[S+] 5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
[S−] 5′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′

The appropriate oligonucleotide primers would be:

Primer 1: GGGGGGGGGG
Primer 2: AAAAAAAAAA

so that if DNA containing [S]

. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

is separated into single strands and its single strands are hybridized to Primers 1 and 2, the following extension reactions can be catalyzed by DNA polymerase in the presence of the four deoxyribonucleoside triphosphates:

3′ 5′
extends ← GGGGGGGGGG Primer 1
. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

original template strand−
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
Primer 2 AAAAAAAAAA → extends
5′ 3′

On denaturation of the two duplexes formed, the products are:

3′ 5′
. . .zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG
newly synthesized long product 1

5′ 3′
. . .zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

-continued

3′ 5′
. . .zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
original template strand−

5′ 3′
AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
newly synthesized long product 3

If these four strands are allowed to rehybridize with Primers 1 and 2 in the next cycle, agent for polymerization will catalyze the following reactions:

Primer 2 5′ AAAAAAAAAA ——————→ extends to here
3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly synthesized long product 1

extends ←—————— GGGGGGGGGG 5′ Primer 1
5′ . . .zzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3′
original template strand+

Primer 2 5′ AAAAAAAAAA ——————→ extends
3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzz . . . 5′
original template strand−

extends to here ←—————— GGGGGGGGGG 5′ Primer 1
5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
newly synthesized long product 2

If the strands of the above four duplexes are separated, the following strands are found:

5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
newly synthesized [S+]

3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
first cycle synthesized long product 1

3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly synthesized long product 1

5′ . . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzz . . . 3′
original template strand+

5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
newly synthesized long product 2

3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . . 5′
original template strand−

3′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly synthesized [S−]

5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
first cycle synthesized long product 2

It is seen that each strand which terminates with the oligonucleotide sequence of one primer and the complementary sequence of the other is the specific nucleic acid sequence [S] that is desired to be produced.

The steps of this process can be repeated indefinitely, being limited only by the amount of Primers 1 and 2, the agent for polymerization and nucleotides present. For detection, the number of cycles used is that required to produce a detectable signal, an amount which will depend, e.g., on the nature of the sample. For example, if the sample is pure or diluted, fewer cycles may be required than if it is a complex mixture. If the sample is human genomic DNA, preferably the number of cycles is from about 10–30.

The amount of original nucleic acid remains constant in the entire process, because it it not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species. This is illustrated in the following table, which indicates the relative amounts of the species theoretically present after n cycles, assuming 100% efficiency at each cycle:

	Number of Double Strands After 0 to n Cycles		
Cycle Number	Template	Long Products	Specific Sequence [S]
0	1	—	—

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-continued

Cycle Number	Number of Double Strands After 0 to n Cycles: Template	Long Products	Specific Sequence [S]
1	1	1	0
2	1	2	1
3	1	3	4
5	1	5	26
10	1	10	1013
15	1	15	32,752
20	1	20	1,048,555
n	1	n	$(2^n - n - 1)$

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

The method herein may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard method of recombinant DNA technology.

Normally, such cloning would either involve direct ligation into a vector or the addition of oligonucleotide linkers followed by restriction enzyme cleavage. Both of these methods involve, however, the inefficient blunt-end ligation reaction. Also, neither technique would control for the orientation or multiplicity of insertion of the amplified product into the cloning vector.

The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture if the original template DNA contains multiple target sequences, such as in a heterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs produced by the amplification reaction. In such modification the same or different restriction sites are incorporated at the 5′ ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate enzymes, the amplified product can then be easily inserted into plasmid or viral vectors and cloned. This cloning allows the analysis or expression of individual amplified products, not a mixture.

Although the same restriction site can be used for both primers, the use of different sites allows the insertion of the product into the vector with a specific orientation and suppresses multiple insertions as well as insertions arising from amplifications based on only one of the two primers. The specific orientation is useful when cloning into single-strand sequencing vectors, when single-strand hybridization probes are used, or when the cloned product is being expressed.

One method to prepare the primers is to choose a primer sequence which differs minimally from the target sequence. Regions in which each of the primers is to be located are screened for homology to restriction sites appropriate to the desired vector. For example, the target sequence "CAGTATCCGA . . . " differs by only one base from one containing a BamHI site. A primer sequence is chosen to match the target exactly at its 3′ end, and to contain the altered sequence and restriction site near its 5′ end (for example, "CAGgATCCGA . . . ", where the lower case letter symbolizes a mismatch with the target sequence). This minimally altered sequence will not interfere with the ability of the primer to hybridize to the original target sequence and to initiate polymerization. After the first amplification cycle the primer is copied, becomes the target, and matches exactly with new primers. After the amplification process, the products are cleaved with the appropriate restriction enzymes, optionally separated from inhibitors of ligation such as the nucleotide triphosphates and salts by passing over a desalting column or molecular weight chromatography column, and inserted by ligation into a cloning vector such as bacteriophage M13. The gene may then be sequenced and/or expressed using well known techniques.

The second method for preparing the primers involves taking the 3′ end of the primers from the target sequence and adding the desired restriction site(s) to the 5′ end of the primer. For the above example, a HindIII site could be added to make the sequence "cgaagctt-CAGTATCCGA . . . ", where lower case letters are as described above. The added bases would not contribute to the hybridization in the first cycle of amplification, but would match in subsequent cycles. The final amplified products are then cut with restriction enzyme(s) and cloned and expressed as described above. The gene being amplified may be, for example, human beta-hemoglobin or the human HLA DQ, DR or DP-α and -β genes.

In addition, the process herein can be used for in vitro mutagenesis. The oligodeoxyribonucleotide primers need not be exactly complementary to the DNA sequence which is being amplified. It is only necessary that they be able to hybridize to the sequence sufficiently well to be extended by the polymerase enzyme or by whatever other inducing agent is employed. The product of a polymerase chain reaction wherein the primers employed are not exactly complementary to the original template will contain the sequence of the primer rather than the template, thereby introducing as in vitro mutation. In further cycles this mutation will be amplified with an undiminished efficiency because no further mispaired primings are required. The mutant thus produced may be inserted into an appropriate vector by standard molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers so as to induce further sequence changes. In this way a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last in a minor way, but from the original DNA source sequence in an increasingly major way. In this manner changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

In addition, the primer can contain as part of its sequence a non-complementary sequence provided that a sufficient amount of the primer contains a sequence which is complementary to the strand to be amplified. For example, a nucleotide sequence which is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5′ end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new

template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

Moreover, the process herein may be used to synthesize a nucleic acid fragment from an existing nucleic acid fragment which is shorter than its product (called the core segment) using certain primers the 3′ ends of which are complementary to or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the original shorter nucleic acid fragments, and the 5′ ends of which primers contain sequence information to be appended to the core segment. This process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to the 3′ end of each strand of said existing fragment such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating tthe single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the product of step (d).

Steps (b) and (c) are repeated as often as necessary, usually at least 5 times, to produce the required amount of the full-length double-stranded product to synthesize the final product (i.e., the effective amount). In addition, the core segment may be obtained as the product of a previous amplification cycle. The product produced in step (d) may be purified before a new cycle of extension and amplification, or used directly by employing the reaction mixture containing the product.

If the 3′ ends of the primers are not exactly complementary to the 3′ ends of the single strands of the original shorter nucleic acid, the core fragment of the product will not be exactly the same as the sequence information resident in the original shorter nucleic acid. Therefore, mutants of the original nucleic acid may be made by using primers which are substantially complementary at their 3′ ends to the 3′ ends of the single strands of the original shorter nucleic acid.

If restriction site linkers are incorporated into the primers, then the amplified double-stranded products can be digested with the appropriate restriction enzymes and ligated directly into an M13 vector for rapid cloning and sequencing. The M13 plaques containing the specific amplified target sequences can be identified by hybridizing plaque lift filters with a probe specific for the target sequence.

The method herein may also be used to enable detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirble.

For purposes of this invention genetic diseases may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, α-thalassemia, β-thalassemia, and the like. Sickle cell anemia can be readily detected via oligomer restriction analysis or a RFLP-like analysis following amplification of the appropriate DNA sequence by the present method. α-Thalassemia can be detected by the absence of a sequence, and β-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a mutation which causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA from, e.g., amniotic fluid containing a very low level of the desired sequence is amplified, cut with a restriction enzyme, and analyzed via a Southern blotting technique. The use of non-radioactive probes is facilitated by the high level of the amplified signal.

In another embodiment a small sample of DNA may be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by restriction and electrophoretic separation or any other appropriate method. An example of this technique in a model system is demonstrated in FIG. **5**.

In a further embodiment, demonstrated in a model system in FIG. **3**, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be detected by an appropriate method.

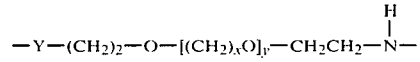
A practical application of this technique can be illustrated by its use in facilitating the detection of sickle cell anemia via the oligomer restriction technique described

herein and in copending U.S. application Ser. No. 716,982 filed Mar. 27, 1985. Sickle cell anemia is a hemoglobin disease which is caused by a single base pair change in the sixth codon of the β-globin gene. FIG. 6 illustrates the sequences of normal and sickle cell β-globin genes in the region of their polymorphism, where the single bars mark the location of a DdeI site present only in the normal gene and where the double bars mark the location of a HinfI site which is non-polymorphic and thus present in both the normal and sickle cell alleles. FIG. 7 illustrates the process of oligomer restriction of normal β-globin DNA using a probe spanning both restriction sites and labeled where the asterisk appears. (The probe is preferably labeled at the end which is fewer base pairs from the restriction site than the other end of the probe.) The DNA, amplified as provided herein, is denatured and annealed to the labeled probe. The amplification may be carried out at elevated temperatures (35°–40° C.) in the presence of dimethyl sulfoxide to minimize formation of secondary structure. The enzyme DdeI cleaves the DNA at the reformed DdeI site and generates a labeled octamer. Under the conditions used in the test the octamer is short enough to dissociate from the duplex. The subsequent addition of the enzyme HinfI has no effect on the now single-stranded octamer. FIG. 8 illustrates the same process applied to the sickle cell allele of β-globin DNA. The enzyme DdeI cannot cleave the duplex formed by the amplified DNA and the labeled probe because of the A-A base pair mismatch. The enzyme HinfI, however, does restrict the hybrid and a labeled trimer is produced. In practice the method can diagnose the DNA of an individual as being either homozygous for the wild type, homozygous for the sickle type or a heterozygous carrier of the sickle cell trait, since a specific signal is associated with the presence of eithr allele. Use of this above-described method to amplify the pertinent sequence allows for a rapid analysis of a single copy gene using a probe with only a single ^{32}P label.

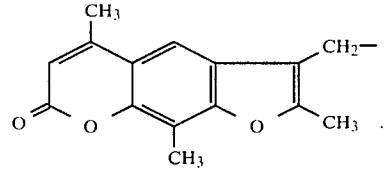
Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism. These include bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis viruses; and parasites, such as the Plasmodium responsible for malaria. U.S. Pat. No. 4,358,535 issued to Falkow describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A problem inherent in the Falkow procedure is that a relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these may constitute only a very small fraction of the total DNA in the sample. Specific amplification of suspected sequences prior to immobilization and hybridization detection of the DNA samples could greatly improve the sensitivity and specificity of these procedures.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as described in EP No. 63,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of specific DNA amplification by the present method and the use of stably labeled probes could provide the convenience and sensitivity required to make the Falkow and Ward procedures useful in a routine clinical setting.

In addition, the probe may be a biotinylated probe in which the biotin is attached to a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4. The spacer arm is in turn attached to a psoralen moiety of the formula:



The psoralen moiety intercalates into and crosslinks a "gapped circle" probe as described by Courage-Tebbe et al., *Biochim. Biophys. Acta,* 697 (1982) 1–5, wherein the single-stranded hybridization region of the gapped circle spans the region contained in the primers. The details of this biotinylation and dot blot procedure are described more fully in commonly assigned copending U.S. application Ser. Nos. 683,263 filed Dec. 18, 1984 and 791,332 filed Oct. 25, 1985, the disclosures of which are incorporated herein by reference.

The amplification process can also be utilized to produce sufficient quantities of DNA from a single copy human gene such that detection by a simple non-specific DNA stain such as ethidium bromide can be employed so as to make a DNA diagnosis directly.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphism which may not be associated with any pathological state.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In these examples all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

A 25 base pair sequence having the nucleotide sequence

5′ CCTCGGCACCGTCACCCTGGATGCT 3′

3′ GGAGCCGTGGCAGTGGGACCTACGA 5′

contained on a 47 base pair FokI restriction fragment of pBR322 obtainable from ATCC was prepared as follows. A FokI digest of pBR322 containing the 47-bp fragment was produced by digesting pBR322 with FokI in accordance with the conditions suggested by the supplier, New England Biolabs Inc. The primers which were utilized were 5′ d(CCTCGGCACCG) 3′ and 5′ d(AGCATCCAGGGTG) 3′, and were prepared using conventional techniques. The following ingredients were added to 33 μl of buffer which consisted of 25 mM potassium phosphate, 10 mM magnesium chloride and 100 mM sodium chloride at pH 7.5: 2433 pmoles of each of the primers described above, 2.4 pmoles of the FokI digest of pBR322, 12 nmoles of dATP, 22 nmoles of dCTP, 19 nmoles of dGTP and 10 nmoles of TTP.

The mixture was heated to 85° C. for five minutes and allowed to cool to ambient temperature. Five units of the Klenow fragment of *E. coli* DNA polymerase I were added and the temperature was maintained for 15 minutes. After that time, the mixture was again heated to 85° C. for five minutes and allowed to cool. Five units of the Klenow fragment were again added and the reaction was carried out for 15 minutes. The heating, cooling and synthesis steps were repeated eleven more times.

After the final repetition, a 5 μl aliquot was removed from the reaction mixture. This was heated to 85° C. for three minutes and allowed to cool to ambient temperature. 12.5 pmoles of α-P^{32}-deoxycytidine triphosphate and 5 units of Klenow fragment were added and the reaction was allowed to proceed for 15 minutes. The labeled products were examined by polyacrylamide gel electrophoresis. The FokI digest was labeled in a similar fashion and served as a control and molecular weight markers. The only heavily labeled band visible after the 13 cycles was the intended 25 base pair sequence.

EXAMPLE 2

The desired sequence to be amplified was a 94 base pair sequence contained within the human beta-globin gene and spanning the MstII site involved in sickle cell anemia. The sequence has the nucleotide sequence shown in FIG. **1**.

I. Synthesis of Primers

The following two oligodeoxyribonucleotide primers were prepared by the method described below:

5′ CACAGGGCAGTAACG 3′ Primer A

and

5′ TTTGCTTCTGACACA 3′ Primer B

Automated Synthesis Procedures: The diethylphosphoramidites, synthesized according to Beaucage and Caruthers (*Tetrahedron Letters* (1981) 22:1859–1862), were sequentially condensed to a nucleotide derivatized controlled pore glass support using a Biosearch SAM-1. The procedure included detritylation with trichloroacetic acid in dichlormethane, condensation using benzotriazole as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine. Cycle time was approximately 30 minutes. Yields at each step were essentially quantitative and were determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

Oligodeoxyribonucleotide Deprotection and Purification Procedures: The solid support was removed from the column and exposed to 1 ml concentrated ammonium hydroxide at room temperature for four hours in a closed tube. The support was then removed by filtration and the solution containing the partially protected oligodeoxyribonucleotide was brought to 55° C. for five hours. Ammonia was removed and the residue was applied to a preparative polyacrylamide gel. Electrophoresis was carried out at 30 volts/cm for 90 minutes after which the band containing the product was identified by UV shadowing of a fluorescent plate. The band was excised and eluted with 1 ml distilled water overnight at 4° C. This solution was applied to an Altech RP18 column and eluted with a 7–13% gradient of acetonitrile in 1% ammonium acetate buffer at pH 6.0. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

Characterization of Oligodeoxyribonucleotides: Test aliquots of the purified oligonucleotides were ^{32}P labeled with polynucleotide kinase and γ-^{32}P-ATP. The labeled compounds were examined by autoradiography of 14–20% polyacrylamide gels after electrophoresis for 45 minutes at 50 volts/cm. This procedure verifies the molecular weight. Base composition was determined by digestion of the oligodeoxyribonucleotide to nucleosides by use of venom diesterase and bacterial alkaline phosphatase and subsequent separation and quantitation of the derived nucleosides using a reverse phase HPLC column and a 10% acetonitrile, 1% ammonium acetate mobile phase.

II. Source of DNA

A. Extraction of Whole Human Wild-Type DNA

Human geonomic DNA homozygous for normal β-globin was extracted from the cell line Molt4 (obtained from Human Genetic Mutant Cell Repository and identified as GM2219c) using the technique described by Stetler et al., *Proc. Nat. Acad. Sci.* USA (1982), 79:5966–5970.

B. Construction of Cloned Globin Genes

A 1.9 kb BamHI fragment of the normal β-globin gene was isolated from the cosmid pFC11 and inserted into the BamHI site of pBR328 (Soberon, et al., Gene (1980) 9:287–305). This fragment, which encompasses the region that hybridizes to the synthetic 40-mer probe, includes the first and second exons, first intron, and 5′ flanking sequences of the gene (Lawn et al., *Cell* (1978), 15:1157–1174). This clone was designated pBR328:HbA and deposited under ATCC No. 39,698 on May 25, 1984.

The corresponding 1.9 kb BamHI fragment of the sickle cell allele of β-globin was isolated from the cosmid pFC12 and cloned as described above. This clone was designated pBR328:HbS and deposited under ATCC No. 39,699 on May 25, 1984.

Each recombinant plasmid was transformed into and propagated in *E. coli* MM294 (ATCC No. 39,607).

C. Digestion of Cloned Globin Genes with MstII

A total of 100 μg each of pBR328:HbA and pBR328:HbS were individually digested with 20 units of MstII (New England Biolabs) for 16 hours at 37° C. in 200 μl of 150 mM NaCl, 12 mM Tris HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 100 μg/ml bovine serum albumin (BSA). The products are designated pBR328:HbA/MstII and pBR328:HbS/MstII, respectively.

III. Polymerase Chain Reaction

To 100 μl of buffer consisting of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 was added 2 μl of a solution containing 100 picomoles of Primer A (of the sequence d(CACAGGGCACTAACG)). 100 picomoles of Primer B (of the sequence d(TTTGCTTCTGACACA)) and 1000 picomoles each of dATP, dCTP, dGTP and TTP. In addition, one of the following sources of DNA described above was added:

10 μg whole human wild-type DNA (Reaction I)

0.1 picomole pBR328:HbA (Reaction II)

0.1 picomole pBR328:HbS (Reaction III)

0.1 picomole pBR328:HbA/MstII (Reaction IV)
0.1 picomole pBR328:HbS/MstII (Reaction V)
No target DNA (Reaction VI)

Each resulting solution was heated to 100° C. for four minutes and allowed to cool to room temperature for two minutes, whereupon 1 μl containing four units of Klenow fragment of *E. coli* DNA polymerase was added. Each reaction was allowed to proceed for 10 minutes, after which the cycle of adding the primers, nucleotides and DNA, heating, cooling, adding polymerase, and reacting was repeated nineteen times for Reaction I and four times for Reactions II–VI.

Four microliter aliquots of Reactions I and II removed before the first cycle and after the last cycle of each reaction were applied to a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours, transferred to a nylon membrane serving as solid phase support and probed with a 5′-^{32}P-labeled 40 pb synthetic fragment, prepared by standared techniques, of the sequence

5′d(TCCTGAGGAGAAGTCTGCCGT-
TACTGCCCTGTGGGGCAAG)3′

in 30% formamide, 3×SSPE, 5×Denhardt's, 5% sodium dodecyl sulfate at pH 7.4. FIG. **2** is an autoradiograph of the probed nylon membrane for Reactions I and II. Lane 1 is 0.1 picomole of a 58-bp synthetic fragment control one strand of which is complementary to the above probe. Lane 2 is 4 μl of Reaction I prior to the first amplification cycle. Lane 3 is 4 μl of Reaction I after the 20th amplification cycle. Lane 4 is 4 μl of Reaction II after five amplification cycles. Lane 5 is a molecular weight standard consisting of a FokI (New England Biolabs) digest of pBR322 (New England Biolabs) labeled with alpha-^{32}P-dNTPs and polymerase. Lane 3 shows that after twenty cycles the reaction mixture I contained a large amount of the specific sequence of the proper molecular weight and no other detectable products. Reaction mixture II after five cycles also contained this product, as well as the starting nucleic acid and other products, as shown by Lane 4.

To 5.0 μl aliquots of Reactions II–VI after the fourth cycle were added 5 pmoles of each primer described above. The solutions were heated to 100° C. for four minutes and allowed to equilibrate to room temperature. Three pmoles each of alpha-^{32}P-dATP, alpha-^{32}P-dCTP, alpha-^{32}P-dGTP and alpha-^{32}P-TTP and four units of Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Four μl aliquots of Reactions II–VI were loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours after which autoradiography was performed.

FIG. **3** is an autoradiograph of the electrophoresis. Lane 1 is a molecular weight standard, Lane 2 is Reaction II, Lane 3 is Reaction III, Lane 4 is Reaction IV and Lane 5 is Reaction V. Another lane for Reaction VI with no DNA as control had no images in any of the lanes. It can be seen from the figure that the 94-bp fragment predicted from the target DNA was present only where intact β-globin DNA sequences were available for amplification, i.e., pBR328:HbA (Lane 2), pBR328:HbS (Lane 3) and pBR328:HbS(MstII (Lane 5). MstII digestion cuts pBR328:HbA in the 94-mer sequence rendering it incapable of being amplified, and the 94-mer band does not appear in Lane 4. In contrast, the 94-mer sequence in pBR328:HbS does not cut when the plasmid is digested with MstII and thus is available for amplification as shown in Lane 5.

FIG. **4** illustrates the chain reaction for three cycles in amplifying the 94-bp sequence. PC01 and PC02 are Primers A and B. The numbers on the right indicate the cycles, whereas the numbers on the left indicate the cycle number in which a particular molecule was produced.

EXAMPLE 3

This example illustrates amplification of a 110 bp sequence spanning the allelic MstII site in the human hemoglobin gene.

A total of 1.0 microgram whole human DNA, 100 picomoles d(ACACAACTGTGTTCACTAGC) and 100 picomoles d(CAACTTCATCCACGTTCACC), the primers having been prepared by the technique of Example 2, were dissolved in 100 μl of a solution which was:

1.5 mM in each of the four deoxyribonucleoside triphosphates
30 mM in Tris acetate buffer at pH 7.9
60 mM in sodium acetate
10 mM in magnesium acetate
0.25 mM in dithiothreitol

The solution was heated to 100° C. for one minute and brought rapidly to 25° C. for one minute, after which was added 2.5 units Klenow fragment of DNA polymerase. The polymerase reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding Klenow, and reacting was repeated as often as desired.

With 70% efficiency at each cycle, 15 cycles resulted in the synthesis of 1.4 femtomoles of the desired 110 bp fragment of the β-globin gene.

EXAMPLE 4

This example illustrates amplification of a 240 bp sequence spanning the allelic MstII site in the human hemoglobin gene. This sequence contains NcoI, HinfI and MstII restriction sites.

To 100 μl of a mixture of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 containing 0.1 pmole pBR328:HbA was added 2 μl of Solution A containing:

100 pmoles d(GGTTGGCCAATCTACTC-CCAGG) primer
100 pmoles d(TAACCTTGATAC-CAACCTGCCC) primer
1000 pmoles each of dATP, dCTP, dGTP and TTP

The two primers were prepared by the technique described in Example 2. The solution was heated to 100° C. for four minutes and allowed to cool in ambient air for two minutes, after which was added 1 μl containing four units Klenow fragment of *E. coli* DNA polymerase. The reaction was allowed to proceed for 10 minutes after which the cycle of solution A addition, heating, cooling, adding polymerase, and reacting was repeated three times. To a 5.0 μl aliquot of the reactions was added 5 picomoles of each oligonucleotide primer described above. The solution was heated to 100° C. for four minutes and allowed to come to ambient temperature, after which 3 picomoles each of the alpha-^{32}P-labeled deoxyribonucleoside triphosphates and 4 units Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given

above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Two μl aliquots were digested with NcoI, MstII, or HinfI and loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours and autoradiography was performed. FIG. 5 illustrates the autoradiograph of the electrophoresis, where Lane 1 is the molecular weight standard, Lane 2 is without digestion with enzyme (240 bp intact), Lane 3 is digestion with NcoI (131 and 109 bp), Lane 4 is digestion with MstII (149 and 91 bp), and Lane 5 is digestion with HinfI (144 and 96 bp). The autoradiograph is consistent with the amplification of the 240 bp sequence.

EXAMPLE 5

This example illustrates use of the process herein to detect sickle cell anemia by sequential digestion.

Synthesis and Phosphorylation of Oligodeoxyribonucleotides

A labeled DNA probe, RS06, of the sequence:

5′ *CTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGG 3′

where * indicates the label, and an unlabeled blocking oligomer, RS10, of the sequence:

3′ GACAGAGGTCACCTCTTCAGACGGCAATGACGGGACACCC 5′

which has three base pair mismatches with RS06 were synthesized according to the procedures provided in Example 2(I). The probe RS06 was labeled by contacting five pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and 50 pmole γ-^{32}P-ATP (New England Nuclear, about 7200 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM spermine, and 2.5 mM dithiothreitol for 90 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 464–465 over a 1 ml Bio Gel P-4 spin dialysis column from Bio-Rad equilibrated with Tris-EDTA (TE) buffer (10 mM Tris buffer, 0.1 mM EDTA, pH 8.0). The labeled probe was further purified by electrophoresis on a 18% polyacrylamide gel (19:1 acrylamide:BIS, Bio-Rad) in Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 500 vhr. After localization by autoradiography, the portion of the gel containing the labeled probe was excised, crushed and eluted into 0.2 ml TE buffer overnight at 4° C. TCA precipitation of the reaction product indicated that the specific activity was 4.9 Ci/mmole and the final concentration was 20 pmole/ml.

The labeled RS10 blocking oligomer was used at a concentration of 200 pmole/ml.

Isolation of Human Genomic DNA from Cell Lines

High molecular weight genomic DNA was isolated from the lymphoid cell lines Molt4, SC-1 and GM2064 using essentially the method of Stetler et al., *Proc. Natl. Acad. Sci.* USA (1982), 79, 5966–5970 (for Molt4) and Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 280–281.

Molt4 (Human Mutant Cell Repository, GM2219C) is a T cell line homozygous for normal β-globin, and SC-1, deposited with ATCC on Mar. 19, 1985, is an EBV-transformed B cell line homozygous for the sickle cell allele. GM2064 (Human Mutant Cell Repository, GM2064) was originally isolated from an individual homozygous for hereditary persistence of fetal hemoglobin (HPFH) and contains no beta- or delta-globin gene sequences. All cell lines were maintained in RPMI-1640 with 10% fetal calf serum.

Isolation of Human Genomic DNA from Clinical Blood Samples

A clinical blood sample designated CH12 from a known sickle cell carrier (AS) was obtained from Dr. Bertram Lubin of Children's Hospital in Oakland, Calif. Genomic DNA was prepared from the buffy coat fraction, which is composed primarily of peripheral blood lymphocytes, using a modification of the procedure described by Nunberg et al., *Proc. Nat. Acad. Sci. USA*, 75, 5553–5556 (1978).

The cells were resuspended in 5 ml Tris-EDTA-NaCl (TEN) buffer (10 mM Tris buffer pH 8, 1 mM EDTA, 10 mM NaCl) and adjusted to 0.2 mg/ml proteinase K, 0.5% SDS, and incubated overnight at 37° C. Sodium perchlorate was then added to 0.7M and the lysate gently shaken for 1–2 hours at room temperature. The lysate was extracted with 30 ml phenol/chloroform (1:1), then with 30 ml chloroform, and followed by ethanol precipitation of the nucleic acids. The pellet was resuspended in 2 ml of TE buffer and RNase A added to 0.005 mg/ml. After digestion for one hour at 37° C., the DNA was extracted once each with equal volumes of phenol, phenol/chloroform, and chloroform, and ethanol precipitated. The DNA was resuspended in 0.5 nm.

Polymerase Chain Reaction to Amplify Selectively β-Globin Sequences

Two micrograms of genomic DNA was amplified in an initial 100 μl reaction volume containing 10 mM Tris buffer (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 150 pmole of Primer A of the sequence d(CACAGGGCACTAACG), and 150 pmole of Primer B of the sequence d(CTTTGCTTCTGACACA) and overlayed with about 100 μl mineral oil to prevent evaporation.

Each DNA sample underwent 15 cycles of amplification where one cycle is composed of three steps:

(1) Denature in a heat block set at 95° C. for two minutes.

(2) Transfer immediately to a heat block set at 30° C. for two minutes to allow primers and genomic DNA to anneal.

(3) Add 2 μl of a solution containing 5 units of the Klenow frgment of *E. coli* DNA polymerase I (New England Biolabs), 1 nmole each of dATP, dCTP, dGTP and TTP, in a buffer composed of 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 4 mM dithiothreitol. This extension reaction was allowed to proceed for 10 minutes at 30° C.

After the final cycle, the reaction was terminated by heating at 95° C. for two minutes. The mineral oil was extracted with 0.2 ml of chloroform and discarded. The final reaction volume was 130 μl.

Hybridization/Digestion of Amplified Genomic DNA with Probes and DdeI/HinfI

Forty-five microliters of the amplified genomic DNA was ethanol precipitated and resuspended in an equal volume of TE buffer. Ten microliters (containing the

pre-amplification equivalent of 154 ng of genomic DNA) was dispensed into a 1.5 ml Microfuge tube and 20 μl of TE buffer to a final volume of 30 μl. The sample was overlayed with mineral oil and denatured at 95° C. for 10 minutes. Ten microliters of 0.6M NaCl containing 0.02 pmole of labeled RS06 probe was added to the tube, mixed gently, and immediately transferred to a 56° C. heat block for one hour. Four microliters of unlabeled RS10 blocking oligomer (0.8 pmole) was added and the hybridization continued for an additional 10 minutes at the same temperature. Five microliters of 60 mM $MgCl_2$/0.1% BSA and 1 μl of DdeI (10 units, New England Biolabs) were added and the reannealed DNA was digested for 30 minutes at 56° C. One microliter of HinfI (10 units, New England Biolabs) was then added and incubated for another 30 minutes. The reaction was stopped by the addition of 4 μl 75 mM EDTA and 6 μl tracking dye to a final volume of 61 μl.

The mineral oil was extracted with 0.2 ml chloroform, and 18 μl of the reaction mixture (45 ng genomic DNA) was loaded onto a 30% polyacrylamide mini-gel (19:1, Bio-Rad) in a Hoeffer SE200 apparatus. The gel was electrophoresed at approximately 300 volts for one hour until the bromophenol blue dye front migrated to 3.0 cm off-origin. The top 1.5 cm of the gel was removed and the remaining gel was exposed for four days with one intensification screen at −70° C.

Discussion of Photograph (FIG. 9)

Each lane contains 45 ng of amplified genomic DNA. Lane A contains Molt4 DNA; Lane B, CH12; Lane C, SC-1; and Lane D, GM2064. Molt4 represents the genotype of a normal individual with two copies of the β^A gene per cell (AA), CH12 is a clinical sample from a sickle cell carrier with one β^A and one β^S gene per cell (AS), and SC-1 represents the genotype of a sickle cell individual with two copies of the β^S gene per cell (SS), GM2064, which contains no beta- or delta-globin sequences, is present as a negative control.

As seen in the photograph, the DdeI-cleaved, β^A-specific octamer is present only in those DNA's containing the β^A gene (Lanes A and B), and the HinfI-cleaved, β^S-specific trimer is present only in those DNA's containing the β^S gene (Lanes B and C). The presence of both trimer and octamer (Lane B) is diagnostic for a sickle cell carrier and is distinguishable from a normal individual (Lane A) with only octamer and a sickle cell afflicted individual (Lane C) with only trimer.

As a comparison, repeating the experiment described above using non-amplified genomic, DNA revealed that the amplification increased the sensitivity of detection by at least 1000 fold.

EXAMPLE 6

This example illustrates direct detection of a totally unpurified single copy gene in which human DNA on gels without the need for a labeled probe.

Using the technique described in Example 3, a 110-bp fragment from a sequence in the first exon of the beta-globin gene was amplified from 10 micrograms of whole human DNA after 20 cycles. This 110-bp fragment produced after 20 cycles was easily visualized on gels stained with ethidium bromide.

The sequence was not amplified when it was first cut with the restriction enzyme DdeI unless, as in the beta-globin S allele, the sequence does not contain the restriction site recognized by the enzyme.

EXAMPLE 7

A. A total of 100 fmoles pBR328 containing a 1.9 kb insert from the human beta-globin A allele, 50 nmoles each alpha-32P-dNTP at 500 Ci/mole, and 1 nmole of each of the primers used in Example 3 were dissolved in a solution containing 100 μl 30 mM Tris-acetate at pH 7.9, 60 mM sodium acetate, 100 mM dithiothreitol, and 10 mM magnesium acetate. This solution was brought to 100° C. for two minutes and cooled to 25° C. for one minute. A total of 1 μl containing 4.5 units Klenow fregment of *E. coli* DNA polymerase I and 0.09 units inorganic pyrophosphatase was added to prevent the possible build-up of pyrophosphate in the reaction mixture, and the reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding enzyme, and reacting was repeated nine times. Ten-μl aliquots were removed and added to 1 μl 600 mM EDTA after each synthesis cycle. Each was analyzed on a 14% polyacrylamide gel in 90 mM Tris-borate and 2.5 mM EDTA at pH 8.3 and 24 volts/cm for 2.5 hours. The completed gel was soaked for 20 minutes in the same buffer with the addition of 0.5 μg/ml ethidium bromide, washed with the original buffer, and photographed in UV light using a red filter.

The 110-bp fragment produced was excised from the gel under ultraviolet light and the incorporated ^{32}P counted by Cerenkov radiation. An attempt to fit the data to an equation of the form: pmoles/10 μl=0.01 $[(1+y)^N-yN-1]$, where N represents the number of cycles and y the fractional yield per cycle, was optimal with y=0.619. This indicates that a significant amplification is occurring.

B. The above experiment was repeated except that 100 nmoles of each dNTP was added to a 100 μl reaction, no radiolabel was employed, and aliquots were not removed at each cycle. After 10 cycles the reaction was terminated by boiling for two minutes and rehybridization was performed at 57° C. for one hour. The sequence of the 110-bp product was confirmed by subjecting 8 μl aliquots to restriction analysis by addition of 1 μl bovine serum albumin (25 mg/ml) and 1 μl of the appropriate restriction enzyme (HinfI, MnlI, MstII, NcoI) and by reaction at 37° C. for 15 hours. PAGE was performed as described above.

EXAMPLE 8

This example illustrates the use of different primers to amplify various fragments of pBR328 and 322.

A. The experiment described in Example 7A was repeated except using the following primers: d(TTTGCTTCTGACACAACTGTGTTCAC-TAGC) and d(GCCTCACCACCAACTTCATC-CACGTTCACC) to produce a 130-bp fragment of pBR328.

B. The experiment described in Example 7A was repeated except using the following primers: d(GGTTGGCCAATCTACTCCCAGG) and d(TGGTCTCCTTAAACCTGTCTTG) to produce a 262-bp fragment of pBR328. The reaction time was 20 minutes per cycle.

The experiment described in Example 8B was repeated except that 100 fmoles of an MstII digest of pBR328 containing a 1.9 kb insert from the human beta-globin S allele was used as initial template. This plasmid was cleaved several times by MstII but not inside the sequence to be amplified. In addition, the primers employed were as follows:

Case 3:11-cv-00056-IEG -WVG Document 1-3 Filed 01/10/11 Page 28 of 99

d(GGTTGGCCAATCTACTCCCAGG) and
d(TAACCTTGATACCAACCTGCCC)
to produce a 240-bp fragment.

D. The experiment described in Example 7B was repeated except that 100 fmoles of an NruI digest of pBR322 was used as template, 200 nmoles of each dNTP were used in the 100 μl reaction, and the primers were:
d(TAGGCGTATCACGAGGCCCT) and
d(CTTCCCCATCGGTGATGTCG)
to produce a 500-bp fragment from pBR322. Reaction times were 20 minutes per cycle at 37° C. Final rehybridization was 15 hours at 57° C. Electrophoresis was on a 4% agarose gel.

EXAMPLE 9

This example illustrates the invention process wherein an in vitro mutation is introduced into the amplified segment.

A. A total of 100 fmoles of pBR322 linearized with NruI, 1 nmole each of the primers:
d(CGCATTAAAGCTTATCGATG) and
d(TAGGCGTATCACGAGGCCCT)
designed to produce a 75-bp fragment, 100 nmole each dNTP, in 100 μl 40 mM Tris at pH 8, 20 mM in $MgCl_2$, 5 mM in dithiothreitol, and 5 mg/ml bovine serum albumin were combined. The mixture was brought to 100° C. for one minute, cooled for 0.5 minutes in a water bath at 23° C., whereupon 4.5 units Klenow fragment and 0.09 units inorganic pyrophosphatase were added, and a reaction was allowed to proceed for three minutes. The cycle of heating, cooling, adding enzymes, and reacting was repeated nine times. The tenth reaction cycle was terminated by freezing and an 8-μl aliquot of the reaction mixture was applied to a 4% agarose gel visualized with ethidium bromide.

B. The experiment described in Example 9A was repeated except that the oligonucleotide primers employed were:
d(CGCATTAAAGCTTATCGATG) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT).
Thse primers are designed to produce a 101-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322. These nucleotides represent the sequence of the T7 promoter, which was appended to the 75-bp sequence from pBR322 by using the primer with 20 complementary bases and a 26-base 5′ extension. The procedure required less than two hours and produced two picomoles of the relatively pure 101-bp fragment from 100 fmoles of pBR322.

The T7 promoter can be used to initiate RNA transcription. T7 polymerase may be added to the 101-bp fragment to produce single-stranded RNA.

C. The experiment described in Example 8D was repeated except that the olignonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(CCAGCAAGACGTAGCCCAGC)
to produce a 1000-bp fragment from pBR322.

D. The experiment described in Example 9C was repeated except that the oligonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT)
so as to produce a 1026-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322 and represent the T7 promoter described above. The promoter has been inserted adjacent to a 1000-bp fragment from pBR322.

The results indicate that a primer which is not a perfect match to the template sequence but which is nonetheless able to hybridize sufficiently to be enzymatically extended produces a long product whwich contains the sequence of the primer rather than the corresponding sequence of the original template. The long product serves as a template for the second primer to introduce an in vitro mutation. In further cycles this mutation is amplified with an undiminished efficiency, because no further mispaired primings are required. In this case, a primer which carries a non-complementary extension on its 5′ end was used to insert a new sequence in the product adjacent to the template sequence being copied.

EXAMPLE 10

This example illustrates employing nested sets of primers to decrease the background in the amplification of single copy genes.

Whole human DNA homozygous for the wild-type β-globin allele was subjected to twenty cycles of amplification as follows: A total of 10 μg DNA, 200 picomoles each of the primers:
d(ACACAACTGTGTTCACTAGC) and
d(CAACTTCATCCACGTTCACC)
and 100 nanomoles each dNTP in 100 μl of 30 mM Tris-acetate pH 7.9, 60 mm sodium acetate, 10 mM dithiothreitol, and 10 mM magnesium acetate were heated to 100° C. for one minute, cooled to 25° C. for one minute, and treated with 2 units Klenow fragment for two minutes. The cycle of heating, cooling and adding Klenow as repeated 19 times. A ten-μl aliquot was removed from the reaction mixture and subjected to a further ten cycles of amplification using each of the primers:
d(CAGACACCATGGTGCACCTGACTCCTG)
and
d(CCCCACAGGGCAGTAACG-
GCAGACTTCTCC),
which amplify a 58-bp fragment contained within the 110-bp fragment produced above. This final ten cycles of amplification was accomplished by diluting the 10-μl aliquot into 90 μl of the fresh Tris-acetate buffer described above containing 100 nanomoles each dNTP and 200 pmoles of each primer. Reaction conditions were as above. After ten cycles a 10-μl aliquot (corresponding to 100 nanograms of the original DNA) was applied to a 6% NuSieve (FMC Corp.) agarose gel and visualized using ethidium bromide.

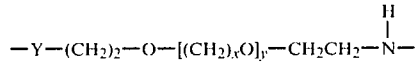
FIG. **10** illustrates this gel illuminated with UV light and photographed through a red filter as is known in the art. Lane 1 is molecular weight markers. Lane 2 is an aliquot of the reaction described above. Lane 3 is an aliquot of a reaction identical to that described above, except that the original wild-type DNA was cleaved with DdeI prior to amplification. Lane 4 is an aliquot of a reaction identical to that described above, except that human DNA homozygous for the sickle betaglobin allele was treated with DdeI prior to amplification (the sickle allele does not contain a DdeI site in the fragment being amplified here). Lane 5 is an aliquot of a reaction identical to that described above, except that salmon sperm DNA was substituted for human DNA. Lane 6 is an aliquot of a reaction identical to that described above, except that the aliquot was treated with DdeI

after amplification (DdeI should convert the 58-bp wild-type product into 27- and 31-bp fragments). Lane 7 is an aliquot of the Lane 4 material treated with DdeI after amplification (the 58-bp sickle product contains no DdeI site).

Detection of a 58-bp fragment representative of a single-copy gene from one microgram of human DNA using only ethidium bromide staining of an agarose gel requires an amplification of about 500,000-fold. This was accomplished by using the two nested sets of oligonucleotide primers herein. The first set amplifies the 110-bp fragment and the inner nested set amplifies a sub-fragment of this product up to the level of convenient detection shown in FIG. **10**. This procedure of using primers amplifying a smaller sequence contained within the sequence being amplified in the previous amplification process and contained in the extension products of the other primers allows one to distinguish the wild-type from the sickle allele at the betaglobin locus without resorting to either radioisotropic or non-radioisotopic probe hybridization methodology such as that of Conner et al., *Proc. Natl. Acad. Sci.* USA, 80:278 (1983) and Leary et al., *Proc. Natl. Acad. Sci.* USA, 80:4045 (1983).

EXAMPLE 11

The present process is expected to be useful in detecting, in a patient DNA sample, a specific sequence associated with an infectious disease such as, e.g., Chlamydia using a biotinylated hybridization probe spanning the desired amplified sequence and using the process described in U.S. Pat. No. 4,358,535, supra. The biotinylated hybridization probe may be prepared by intercalation and irradiation of a partially double-stranded DNA with a 4'-methylene substituted 4,5'-8-trimethylpsoralen attached to biotin via a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4, as described in U.S. Pat. Nos. 4,582,789 issued Apr. 15, 1986 and 4,617,261 issued Oct. 14, 1986, the disclosures of which are incorporated herein by reference. Detection of the biotinyl groups on the probe may be accomplished using a streptavidin-acid phosphatase complex commercially obtainable from Enzo Biochemical using the detection procedures suggested by the manufacturer in its brochure. The hybridized probe is seen as a spot of precipitated stain due to the binding of the detection complex, and the subsequent reaction catalyzed by acid phosphatase, which produces a precipitable dye.

EXAMPLE 12

In this example, the process of Example 7 was basically used to amplify a 119 base pair fragment on the human β-hemoglobin gene using the primers:

5'-CTTCTGcagCAACTGTGTTCACTAGC-3' (GH18)

5'-CACaAgCTTCATCCACGTTCACC-3' (GH19)

where lower case letters denote mismatches from wild-type sequence to create restriction enzyme sites. The full scheme is shown in Table I. Table I illustrates a diagram of the primers GH18 and GH19 which are used for cloning and sequencing a 119-base pair fragment of the human β-globin gene and which are designed to contain internal restriction sites. The start codon ATG is underlined. GH18 is a 26-base oligonucleotide complementary to the negative strand and contains an internal PstI site. GH19 is a 23-base oligonucleotide complementary to the plus strand and contains an internal HindIII recognition sequence. Arrows indicate the direction of extension by DNA polymerase I. The boxed sequences indicate the restriction enzyme recognition sequences of each primer. These primers were selected by first screening the regions of the gene for homology to the PstI and HindIII restriction sites of bacteriophage M13. The primers were then prepared as described in previous examples.

TABLE I

DdeI

GH19

←CCACTTGCACCTAC TTCgAa CAC

CTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTG(+)

GAAGACTGTGTTGACACAAGTGATCGTTGGAGTTTGTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACGGCAATGACGGGACACCCCGTTCCACTTGCACCTACTTCAACCAC(−)

CTTCTG cagCAA CTGTGTTCACTAGC→

GH18

Pst I

5′ CTTCTG cagCAA CTGTGTTCACTAGC 3′ GH18 left linker primer

5′ CAC aAgCTT CATCCACGTTCACC 3′ GH19 roght linker primer

Hind III

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Amplification and Cloning

After twenty cycles of amplification of 1 microgram of human genomic DNA isolated from the cell line Molt 4 as described in Example 2, 1/14th of the reaction product was hybridized to the labeled β-globin specific oligonucleotide probe, RS06, of the sequence 5'-CTGACTCCTGAGGAGAAGTCTGCCGT-TACTGCCCTGTGGG-3' using the methods described above for oligomer restriction. Following solution hybridization, the reaction mixture was treated with DdeI under restriction digestion conditions as described above, to produce an 8-base pair oligonucleotide. The amount of this 8-base pair product is proportional to the amount of amplified product produced. The digestion products were resolved on a 30% polyacrylamide gel and visualized by autoradiography.

Analysis of the autoradiogram revealed that the amplification was comparable in efficiency to that of amplification with primers PC03 (5'-ACACAACTGTGTTCACTAGC-3') and PC04 (5'-CCACTTGCACCTACTTCAAC-3'), which are complementary to the negative and positive strands, respectively, of the wild-type β-globin.

The amplified product was ethanol precipitated to desalt and concentrate the sample, redissolved in a restriction buffer of 10 mM Tris pH 8, 10 mM $MgCl_2$, 1 mM DTT, 100 mM $NaCl_2$, and simultaneously digested with PstI and HindIII. After digestion the sample was desalted with a Centricon 10 concentrator and ligated overnight at 12° C. with 0.3 micrograms of the PstI/-HindIII digested vector M13mp10w, which is publicly available from Boehringer-Mannheim.

The entire ligation mixture was transformed into *E. coli* strain JM103, which is publicly available from BRL in Bethesda, MD. The procedure followed by preparing the transformed strain is described in Messing, J. (1981) *Third Cleveland Symposium on Macromolecules:Recombinant DNA,* ed. A. Walton, Elsevier, Amsterdam, 143–153.

The transformation mixture was plated onto x-gal media for screening via plaque hybridization with nylon filters. The filters were probed with a β-globin-specific oligonucleotide probe RS24 of the sequence 5'-CCCACAGGGCAGTAACGGCAGACTTCTCCT-CAGGAGTCAG-3' to determine the number of β-globin inserts. The filters were then reprobed with the primer PC04 to determine the total number of inserts.

Plating and Screening

Table II summarizes the plating and plaque hybridization data. The filters were probed with the primer PC04 to determine the percentage of inserts resulting from amplification and cloning; 1206 clear plaques (90% of total number of clear plaques) hybridized to the primer. Fifteen plaques hybridized to the β-globin specific probe RS24. The percentage of β-globin positive plaques among the amplified primer-positive plaques is approximately 1%.

TABLE II

Plate No.	Blue Plaques	No Inserts*	Inserts**	β-Globin Inserts
1	28	25	246	1
2	29	18	222	2
3	11	26	180	0
4	24	20	192	5
5	22	27	185	5
6	39	21	181	3
TOTAL	158	132	1206	15

% of plaques containing amplified sequences which contain β-globin insert = 15/1206 × 100 = 1.24%

% of total plaques which contain β-globin insert = 15/1496 × 100 = ca.1%

% of total plaques which contain amplified sequences = 1206/1496 × 100 = 0.8%

*Clear plaques which do not hybridize to primer PC04

**Clear plaques which hybridize to primer PC04

Restriction Enzyme and Southern Blot Analysis

DNAs from phage DNA minipreparation of three β-globin positive and two β-globin negative (but PC04 primer positive) plaques were analyzed by restriction enzyme analysis. MstII digestion of DNA from M13 clones containing the amplified β-globin fragment should generate a characteristic 283 base-pair fragment. Following MstII digestion, the three β-globin positive clones all produced the predicted 283 base pair fragment, while the two clones which were positive only with the primer produced larger fragments.

The gel from this analysis was transferred to a MSI nylon filter and hybridized with a radiolabeled nick-translated β-globin probe prepared by standard nick translation methods as described by Rigby et al., *J. Mol. Biol.* (1977), 113:237–51. The only bands which hybridized to the β-globin probe were the three β-globin positive clones. The two other clones had inserts which did not hybridize to the β-globin probe.

Sequence Analysis

Ten β-globin positive clones which were shown by restriction enzyme analysis to contain the β-globin insert were sequenced using the M13-dideoxy sequencing method. Of the ten clones, nine were identical to the β-globin wild-type sequence. The other clone was identical to the ∂-globin gene which had been shown to be amplified to only a small degree by the β-globin primers.

In conclusion, the modified linker primers were nearly as efficient as the unmodified primers in amplifying the β-globin sequence. The primers were able to facilitate insertion of amplified DNA into cloning vectors. Due to the amplification of other segments of the genome, only 1% of the clones contained hemoglobin sequences.

Nine of the ten clones were found to be identical to the published β-globin sequence, showing that the technique amplifies genomic DNA with high fidelity. One clone was found to be identical with the published ∂-globin sequence, confirming that the primers are specific for the β-globin gene despite their having significant sequence homology with δ-globin.

When cloning was carried out with a 267 base pair fragment of the β-globin gene, cloning was effective only when dimethylsulfoxide was present (10% by volume at 37° C.) in the amplification procedure.

Restriction site-modified primers were also used to amplify and clone and partially sequence the human N-ras oncogene and to clone 240-base pair segments of the HLA DQ-α and DQ-β genes. All of these amplifications were carried out in the presence of 10% by volume dimethylsulfoxide at 37° C. The primers for amplifying HLA DQ-α and DQ-β genes were much more specific for their intended targets than were the β-globin and DR-β primers, which, rather than giving a

discrete band on an ethidium bromide stained agarose gel, produced only a smear. In addition, the HLA DQ-α primers produced up to 20% of clones, with amplified inserts which contained the desired HLA target fragment, whereas 1% of the β-globin clones contained the target sequence. The HLA DQ-α and DQ-β gene cloning was only effective when the DMSO was present and the temperature was elevated.

EXAMPLE 13

This example illustrates the use of the process herein to prepare the TNF gene of 494 base pairs starting from two oligonucleotides of 74 base pairs each.

PRIMERS

The primers employed were prepared by the method described in Example 2 and are identified below, each being 74 mers.

(TN10) 5′-CCTCGTCTACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCGACTATGTGCTCCTCACCCACACCGTCAGCC-3′

(TN11) 5′-GGCAGGGGCTCTTGACGGCAGAGAGGAGGTTCACCTTCTCCTGGTAGGAGATGGCGAAGCGGCTGACGGTGTGG-3′

(LL09) 5′-CCTGGCCAATGGCATGGATCTGAAAGATAACCAGCTGGTGGTGCCAGCAGATGGCCTGTACCTCGTCTACTCCC-3′

(LL12) 5′-CTCCCTGATAGATGGGCTCATACCAGGGCTTGAGCTCAGCCCCCTCTGGGGTGTCCTTCGGGCAGGGGCTCTTG-3′

(TN08) 5′-TGTAGCAAACCATCAAGTTGAGGAGCAGCTCGAGTGGCTGAGCCAGCGGGCCAATGCCCTCCTGGCCAATGGCA-3′

(TN13) 5′-GATACTTGGGCAGATTGACCTCAGCGCTGAGTTGGTCACCCTTCTCCAGCTGGAAGACCCCTCCCTGATAGATG-3′

(LL07) 5′-CCTTAAGCTTATGCTCAGATCATCTTCTCAAAACTCGAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCATC-3′

(TN14) 5′-GCTCGGATCCTTACAGGGCAATGACTCCAAAGTAGACCTGCCCAGACTCGGCAAAGTCGAGATACTTGGGCAGA-3′

OVERALL PROCEDURE

I. Ten cycles of the protocol indicated below were carried out using primers TN10 and TN11, which interact as shown in the diagram below, step (a).

II. A total of 2 μl of the reaction mixture from Part I above was added to the primers LL09 and LL12. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part I as shown in the diagram below, step (b).

III. A total of 2 μl of the reaction mixture from Part II above was added to the primers TN08 and TN13. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part II as shown in the diagram below, step (c).

IV. A total of 2 μl of the reaction mixture from Part III above was added to the primers LL07 and LL14. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part III as shown in the diagram below, step (d).

PROTOCOL

Each reaction contained 100 μl of:

2 mM of each of dATP, dCTP, dGTP and TTP

3 μM of each of the primers used at that step

1×polymerase buffer, (30 mM Tris-acetate, 60 mM Na-acetate, 10 mM Mg-acetate, 2.5 mM dithiothreitol)

Each cycle constituted:

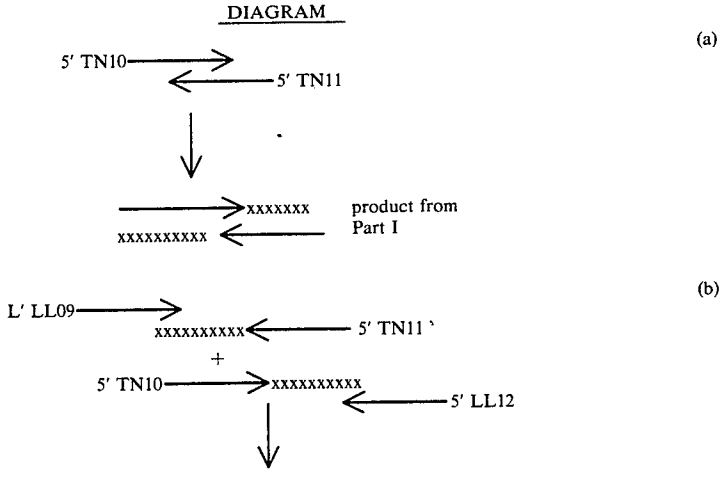
(1) 1 min. in boiling water

(2) 1 min. cooling at room temperature

(3) add 1 μl (5 units) of the Klenow fragment of DNA polymerase

(4) allow the polymerization reaction to proceed for 2 min. For the next cycle start again at step 1.

DIAGRAM



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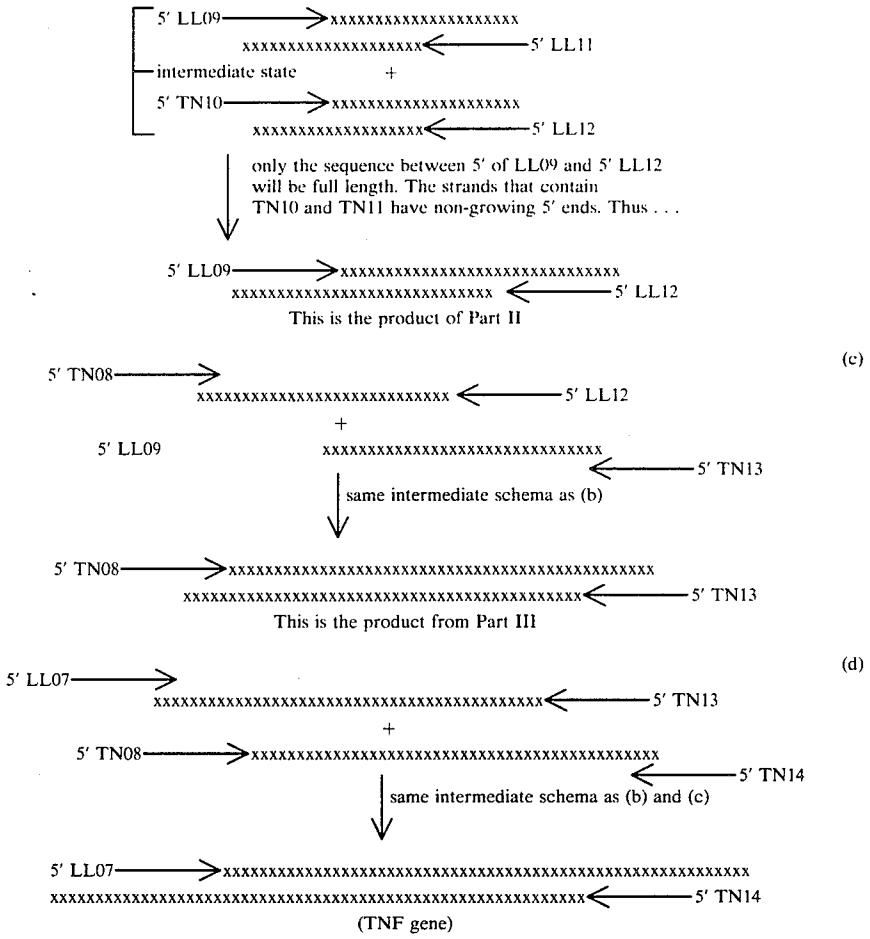
-continued

DIAGRAM



Deposit of Materials

The cell line SC-1 (CTCC #0082) was deposited on Mar. 19, 1985 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 USA, with ATCC Accession No. CRL#8756. The deposit of SC-1 was made pursuant to a contract between the ATCC and the assignee of this patent application, Cetus Corporation. The contract with ATCC provides for permanent availability of the progeny of this cell line to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progency of this cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell line on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same cell line.

In summary, the present invention is seen to provide a process for detecting sequences in nucleic acids by first amplifying one or more specific nucleic acid sequences using a chain reaction in which primer extension products are produced which can subsequently act as templates for further primer extension reactions. The process is especially useful in detecting nucleic acid sequences which are initially present in only very small amounts. Also, the amplification process can be used for molecular cloning.

Other modifications of the above described embodiments of the invention which are obvious to those of skill in the area of molecular biology and related disciplines are intended to be within the scope of the following claims.

What is claimed is:

1. A process for detecting the presence or absence of at least one specific nuclei acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence, under hybridizing conditions such that for each strand of each different sequence to which an oligonucleotide primer is hybridized an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be sufficiently complementary to each strand of each specific sequence to hybridize therewith such

that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present;

(c) treating the sample with olignonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the specific nucleic acid sequence or sequences if present;

(d) adding to the product of step (c) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to said sequence or a mutation thereof; and

(e) determining whether said hybridization has occurred.

2. The process of claim **1**, wherein steps (b) and (c) are repeated at least once.

3. The process of claim **1**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primer(s).

4. The process of claim **1**, wherein said nucleic acid is double stranded and its strands are separated by denaturing before or during step (a).

5. The process of claim **1**, wherein said nucleic acid is single stranded.

6. The process of claim **4**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

7. The process of claim **4**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

8. The process of claim **5**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

9. The process of claim **5**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

10. The process of claim **1**, wherein each primer employed contains a restriction site on its 5′ end which is the same as or different from a restriction site on another primer, and after step (c) and before step (d) the product of step (c) is cleaved with a restriction enzyme specific for each of said restriction sites and the cleaved products are separated from the uncleaved products and used in step (d).

11. The process of claim **1**, wherein the specific nucleic acid sequence contains at least one specific deletion or mutation that causes a genetic disease.

12. The process of claim **11**, wherein the genetic disease is sickle cell anemia.

13. The process of claim **11**, wherein after step (c) and before step (d) the treated sample is cut with a restriction enzyme and electrophoresed and step (e) is accomplished by Southern blot analysis.

14. The process of claim **1**, wherein the specific nucleic acid sequence is contained in a pathogenic organism or is contained in an oncogene.

15. The process of claim **1**, wherein steps (a) and (c) are accomplished using an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, reverse transcriptase wherein the template is RNA or DNA and the extension product is DNA, and an enzyme that after being exposed to a temperature of about 65°–90° C. forms said extension products at the temperature of reaction during steps (a) and (c).

16. A process for detecting the presence or absence of a nucleic acid sequence containing a polymorphic restriction site specific for sickle cell anemia which sequence is suspected of being contained in a sample, which process comprises:

(a) treating the sample, together or separately, with an oligodeoxyribonucleotide primer for each strand, four different nucleoside triphosphates, and an agent for polymerization under hybridizing conditions, such that for each strand of the nucleic acid sequence an extension product of each primer is synthesized which is sufficiently complementary to each strand of the nucleic acid sequence being detected to hybridize therewith and contains the region of the β-globin gene known potentially to contain the mutation that causes sickle cell anemia, wherein said primers are selected such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from the templates on which they are synthesized if the sequence to be detected is present;

(c) treating the product of step (b) with oligodeoxyribonucleotide primers, four different nucleoside triphosphates, and an agent polymerization such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the sequence to be detected if present;

(d) hybridizing said primer extension products of step (c) with a labeled oligodeoxyribonucleotide probe complementary to a normal β-globin gene;

(e) digesting the hybridized mixture from step (d) with a restriction enzyme for the restriction site specific for sickle cell anemia; and

(f) detecting whether the digest contains a restriction fragment correlated with the presence of sickle cell anemia.

17. The process of claim **16**, wherein in step (d) the probe spans DdeI and HinfI restriction sites, in step (e) the restriction enzyme is DdeI, and after step (e) and before step (f) the mixture is digested with restriction enzyme HinfI.

18. The process of claim **16**, wherein in steps (d)–(f) are present a positive control which contains a nucleic acid with the polymorphic restriction site specific for sickle cell anemia and a negative control which does not contain such nucleic acid.

19. A process for synthesizing a nucleic acid fragment from an existing nucleic acid fragment having fewer nucleotides than the fragment being synthesized and two oligonucleotide primers, wherein the nucleic acid being synthesized is comprised of a left segment, a core segment and a right segment, and wherein the core segment is sufficiently complementary to the nucleotide sequence of said existing nucleic acid fragment to hybridize therewith, and the right and left segments represent the nucleotide sequence present in the 5′ ends of the two primers, the 3′ ends of which are complementary to, or sufficiently complementary to hybridize with, the 3′ ends of the single strands produced by separating the strands of said existing nucleic acid fragment, which process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such

that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to the 3′ end of each strand of said existing fragment to hybridize therewith, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generared from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary to, or sufficiently complementary to hybridize with, the 3′ ends of the single strands produced by separating the strands of the product of step (d).

20. The process of claim **19**, wherein steps (b) and (c) are repeated at least five times.

21. The process of claim **20**, wherein the core segment used is the product of step (f).

22. The process of claim **19**, wherein the core fragment used is obtained by the steps comprising:

(a) reacting two oligonucleotides, each of which contain at their 3′ ends a nucleotide sequence which is complementary to the other oligonucleotide at its 3′ end, and which are non-complementary to each other at their 5′ ends, with an agent for polymerization and four nucleoside triphosphates under conditions such that an extension product of each oligonucleotide is synthesized which is complementary to each nucleic acid strand;

(b) separating the extension products from the templates on which they were synthesized to produce single-stranded molecules; and

(c) treating the single-stranded molecules generated from step (b) with the oligonucleotides of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the core fragment.

23. The process of claim **19**, wherein the product of step (d) is purified before step (e).

24. The process of claim **19**, wherein the product of step (d) is not purified before step (e).

25. The process of claim **19**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primers.

26. A process for cloning into bacteriophage M13 a polymorphic genetic sequence on the human HLA DQ, DR or DP Class Ii α and β genes, which process comprises:

(a) treating a genetic sequence of human HLA DQ, DR, or DP Class II α and β genes with one oligonucleotide primer for each strand of said sequence, under conditions such that for each strand an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to each strand to hybridize therewith such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each of said primers contains a restriction site on its 5′ end which is different from the restriction site on the other primer;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, wherein steps (a) and (c) are carried out in the presence of an effective amount of dimethylsulfoxide to amplify sufficiently the amount of sequence produced and at a temperature of 35°–40° C.;

(d) adding to the product of step (c) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(e) ligating the cleaved products into said bacteriophage M13 with a specific orientation.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,683,195
DATED : July 28, 1987
INVENTOR(S) : Mullis et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
After [*] Notice, please replace "The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed." with -- This patent is subject to a terminal disclaimer. --

Signed and Sealed this

First Day of July, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

United States Patent [19]

Mullis et al.

[11] **Patent Number: 4,965,188**

[45] **Date of Patent: * Oct. 23, 1990**

[54] **PROCESS FOR AMPLIFYING, DETECTING, AND/OR CLONING NUCLEIC ACID SEQUENCES USING A THERMOSTABLE ENZYME**

[75] Inventors: **Kary B. Mullis**, La Jolla; **Henry A. Erlich**; **David H. Gelfand**, both of Oakland; **Glenn Horn**, Emeryville; **Randall K. Saiki**, Richmond, all of Calif.

[73] Assignee: **Cetus Corporation**, Emeryville, Calif.

[*] Notice: The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed.

[21] Appl. No.: **63,647**

[22] Filed: **Jun. 17, 1987**

Related U.S. Application Data

[60] Continuation-in-part of Ser. No. 899,513, Aug. 22, 1986, abandoned, which is a continuation-in-part of Ser. No. 839,331, Mar. 13, 1986, abandoned, and a continuation-in-part of Ser. No. 828,144, Feb. 7, 1986, Pat. No. 4,683,195, which is a continuation-in-part of Ser. No. 824,044, Jan. 30, 1986, abandoned, which is a division of Ser. No. 791,308, Oct. 25, 1985, Pat. No. 4,683,202, which is a continuation-in-part of Ser. No. 716,975, Mar. 28, 1985, abandoned.

[51] **Int. Cl.**[5] **C12Q 1/68**; C12P 21/00; C12P 19/34; C12N 15/00

[52] **U.S. Cl.** **435/6**; 435/69.1; 435/91; 435/172.3; 935/17; 935/78

[58] **Field of Search** 435/6, 91, 172.3, 172.1, 435/320, 69.1; 536/27; 935/17, 18, 76, 77, 78; 436/63, 94, 501, 508

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[57] **ABSTRACT**

A process for amplifying any target nucleic acid sequence contained in a nucleic acid or mixture thereof comprises treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers and extending the primers with a thermostable enzyme to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence. The amplified sequence can be readily detected. The steps of the reaction can be repeated as often as desired and involve temperature cycling to effect hybridization, promotion of activity of the enzyme, and denaturation of the hybrids formed.

50 Claims, No Drawings

PROCESS FOR AMPLIFYING, DETECTING, AND/OR CLONING NUCLEIC ACID SEQUENCES USING A THERMOSTABLE ENZYME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part (CIP) of now abandoned U.S. patent application Ser. No. 899,513, filed Aug. 22, 1986, which is a CIP of now abandoned U.S. patent application No. 839,331, filed Mar. 13, 1986, and of U.S. patent application Ser. No. 824.44, filed Jan. 30, 1986, which is a division of U.S. application patent Ser. No. 791,308, filed Oct. 25, 1985, now U.S. Pat. No. 4,683,202, which is a CIP of now abandoned U.S. patent application Ser. No. 716,975, filed Mar. 28, 1985.

FIELD OF THE INVENTION

The present invention relates to a process for amplifying existing nucleic acid sequences if they are present in a test sample and detecting them if present by using a probe. More specifically, it relates to a process for producing any particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present so as to facilitate detection of the sequences, using a thermostable enzyme to catalyze the reaction. The DNA or RNA may be single- or double-stranded, and may be a relatively pure species or a component of a mixture of nucleic acids. The process of the invention utilizes a repetitive reaction to accomplish the amplification of the desired nucleic acid sequence.

DESCRIPTION OF RELATED DISCLOSURES

For diagnostic applications in particular, the target nucleic acid sequence may be only a small portion of the DNA or RNA in question, so that it may be difficult to detect its presence using nonisotopically labeled or end-labeled oligonucleotide probes. Much effort is being expended in increasing the sensitivity of the probe detection systems, but little research has been conducted on amplifying the target sequence so that it is present in quantities sufficient to be readily detectable using currently available methods.

Several methods have been described in the literature for the synthesis of nucleic acids de novo or from an existing sequence. These methods are capable of producing large amounts of a given nucleic acid of completely specified sequence.

One known method for synthesizing nucleic acids de novo involves the organic synthesis of a nucleic acid from nucleoside derivatives. This synthesis may be performed in solution or on a solid support. One type of organic synthesis is the phosphotriester method, which has been utilized to prepare gene fragments or short genes. In the phosphotriester method, oligonucleotides are prepared which can then be joined together to form longer nucleic acids. For a description of this method, see Narang, S.A., et al., *Meth. Enzymol.*, 68, 90 (1979) and U.S. Pat. No. 4,356,270. The patent describes the synthesis and cloning of the somatostatin gene.

A second type of organic synthesis is the phosphodiester method, which has been utilized to prepare a tRNA gene. See Brown, E. L., et al., *Meth. Enzymol.*, 68, 109 (1979) for a description of this method. As in the phosphotriester method, the phosphodiester method involves synthesis of oligonucleotides which are subsequently joined together to form the desired nucleic acid.

Although the above processes for de novo synthesis may be utilized to synthesize long strands of nucleic acid, they are not very practical to use for the synthesis of large amounts of a nucleic acid. Both processes are laborious and time-consuming, require expensive equipment and reagents, and have a low overall efficiency. The low overall efficiency may be caused by the inefficiencies of the synthesis of the oligonucleotides and of the joining reactions. In the synthesis of a long nucleic acid, or even in the synthesis of a large amount of a shorter nucleic acid, many oligonucleotides would need to be synthesized and many joining reactions would be required. Consequently, these methods would not be practical for synthesizing large amounts of any desired nucleic acid.

Methods also exist for producing nucleic acids in large amounts from small amounts of the initial existing nucleic acid. These methods involve the cloning of a nucleic acid in the appropriate host system, where the desired nucleic acid is inserted into an appropriate vector which is used to transform the host. When the host is cultured the vector is replicated, and hence more copies of the desired nucleic acid are produced. For a brief description of subcloning nucleic acid fragments, see Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, pp. 390401 (1982). See also the techniques described in U.S. Pat. Nos. 4,416,988 and 4,403,036.

A third method for synthesizing nucleic acids, described in U.S. Pat. No. 4,293,652, is a hybrid of the above-described organic synthesis and molecular cloning methods. In this process, the appropriate number of oligonucleotides to make up the desired nucleic acid sequence is organically synthesized and inserted sequentially into a vector which is amplified by growth prior to each succeeding insertion.

The present invention bears some similarity to the molecular cloning method; however, it does not involve the propagation of any organism and thereby avoids the possible hazards or inconvenience which this entails. The present invention also does not require synthesis of nucleic acid sequences unrelated to the desired sequence, and thereby the present invention obviates the need for extensive purification of the product from a complicated biological mixture.

European Pat. Publication No. 200,362 published Dec. 10, 1986 discloses a procedure whereby existing nucleic acids may be produced in larger quantities so as to prepare other nucleic acids or to diagnose for the presence of nucleic acids. The amplification and detection process is also described by Saiki et al., *Science*, 230:1350–1354 (1985), and by Saiki et al., *Biotechnology*, 3:1008–1012 (1985). Copending U.S. patent application Ser. No. 899,061 filed Aug. 22, 1986, supra, discloses carrying out an amplification of nucleic acids in the presence of a thermostable enzyme in a heat-conducting block whose temperature is controlled by computer means. Copending U.S. patent application Ser. No. 899,344 filed Aug. 22, 1986, supra, discloses an amplification procedure followed by dot blot analysis using a heatstable enzyme. Now abandoned U.S. application patent Ser. No. 899,241 filed Aug. 22, 1986, supra, discloses purification of a thermostable enzyme, preferably a polymerase from *Thermus aquaticus*.

SUMMARY OF THE INVENTION

The present invention resides in a process for amplifying one or more specific nucleic acid sequences present in a nucleic acid or mixture thereof using primers and a thermostable enzyme. The extension product of one primer when hybridized to the other becomes a template for the production of the desired specific nucleic acid sequence, and vice versa, and the process is repeated as often as is necessary to produce the desired amount of the sequence. The method herein improves the specificity of the amplification reaction, resulting in a very distinct signal of amplified nucleic acid. In addition, the method herein eliminates the need for transferring reagents from one vessel to another after each amplification cycle. Such transferring is not required because the thermostable enzyme will withstand the high temperatures required to denature the nucleic acid strands and therefore does not need replacement. The temperature cycling may, in addition, be automated for further reduction in manpower and steps required to effectuate the amplification reaction.

More specifically, the present invention provides a process for amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, wherein if the nucleic acid is double-stranded, it consists of two separated complementary strands of equal or unequal length, which process comprises:

(a) contacting each nucleic acid strand with four different nucleoside triphosphates and one oligonucleotide primer for each different specific sequence being amplified, wherein each primer is selected to be substantially complementary to different strands of each specific sequence, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, said contacting being at a temperature which promotes hybridization of each primer to its complementary nucleic acid strand.,

(b) contacting each nucleic acid strand, at the same time as or after step (a), with a thermostable enzyme which enables combination of the nucleotide triphosphates to form primer extension products complementary to each strand of each nucleic acid,

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to activate the enzyme, and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each nucleic acid strand template, but not so high (a temperature) as to separate each extension product from its complementary strand template;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the templates on which they were synthesized to produce single-stranded molecules, but not so high (a temperature) as to denature irreversibly the enzyme;

(e) cooling the mixture from step (d) at an effective temperature for an effective time to promote hybridization of each primer to each of the single-stranded molecules produced in step (d); and

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each nucleic acid strand template produced in step (d), but not so high (a temperature) as to separate each extension product from its complementary strand template, wherein steps (e) and (f) are carried out simultaneously or sequentially.

The steps (d), (e) and (f) may be repeated until the desired level of sequence amplification is obtained. The preferred thermostable enzyme herein is a polymerase extracted from *Thermus aquaticus* (Taq polymerase). Most preferably, if the enzyme is Taq polymerase, in step (a) the nucleic acid strands are contacted with a buffer comprising about 1.5–2 mM of a magnesium salt, 150–200 μM each of the nucleotides, and 1 nM of each primer, steps (a), (e) and (f) are carried out at about 45–58° C., and step (d) is carried out at about 90–100° C.

In a preferred embodiment, the nucleic acid(s) are doublestranded and step (a) is accomplished by (i) heating each nucleic acid in the presence of four different nucleoside triphosphates and one oligonucleotide primer for each different specific sequence being amplified, for an effective time and at an effective temperature to denature each nucleic acid, wherein each primer is selected to be substantially complementary to different strands of each specific sequence, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer., and (ii) cooling the denatured nucleic acids to a temperature which promotes hybridization of each primer to its complementary nucleic acid strand.

In other embodiments the invention relates to a process for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, and wherein if the nucleic acid(s) are doublestranded, they each consist of two separated complementary strands of equal or unequal length, which process comprises steps (a) to (f) mentioned above, resulting in amplification in quantity of the specific nucleic acid sequence(sl, if present;

(g) adding to the product of step (f) a labeled oligonucleotide probe, for each sequence being detected, capable of hybridizing to said sequence or to a mutation thereof; and

(h) determining whether said hybridization has occurred.

In yet another embodiment, the invention relates to a process for detecting the presence or absence of at least one nucleotide variation in sequence in one or more nucleic acids contained in a sample, wherein if the nucleic acid is double-stranded it consists of two separated complementary strands of equal or unequal length, which process comprises steps (a)–(f) mentioned above, wherein steps (d), (e) and (f) are repeated a sufficient number of times to result in detectable amplification of the nucleic acid containing the sequence, if present;

(g) affixing the product of step (f) to a membrane,

(h) treating the membrane under hybridization conditions with a labeled sequence-specific oligonucleotide probe capable of hybridizing with the amplified nucleic acid sequence only if a sequence of the probe is complementary to a region of the amplified sequence; and

(i) detecting whether the probe has hybridized to an amplified sequence in the nucleic acid sample.

If the sample comprises cells, preferably they are heated before step (a) to expose the nucleic acids therein

to the reagents. This step avoids extraction of the nucleic acids prior to reagent addition.

In a variation of this process, the primer(s) and/or nucleotide triphosphates are labeled so that the resulting amplified sequence is labeled. The labeled primer(s) and/or nucleotide triphosphate(s) can be present in the reaction mixture initially or added during a later cycle. The sequence-specific oligonucleotide (unlabeled) is affixed to a membrane and treated under hybridization conditions with the labeled amplification product so that hybridization will occur only if the membrane-bound sequence is present in the amplification product.

In yet another embodiment, the invention herein relates to a process for cloning into a cloning vector one or more specific nucleic acid sequences contained in a nucleic acid or a mixture of nucleic acids, which nucleic acid(s) when double-stranded consist of two separated complementary strands, and which nucleic acid(s) are amplified in quantity before cloning, which process comprises steps (a)–(f) mentioned above, with steps (d), (e) and (f) being repeated a sufficient number of times to result in detectable amplification of the nucleic acid(s) containing the sequence(s);

(g) adding to the product of step (f) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(h) ligating the cleaved product(s) of step (g) containing the specific sequence(s) to be cloned into one or more cloning vectors containing a promoter and a selectable marker.

In a final embodiment, the invention herein relates to a process for cloning into a cloning vector one or more specific nucleic acid sequences contained in a nucleic acid or mixture of nucleic acids, which nucleic acid(s), when double-stranded, consist of two separated complementary strands of equal or unequal length which nucleic acid(s) are amplified in quantity, before cloning, which process comprises steps (a)–(f) mentioned above, with steps (d), (e) and (f) being repeated a sufficient number of times to result in effective amplification of the nucleic acid(s) containing the sequence(s) for blunt-end ligation into one or more cloning vectors; and

(g) ligating the amplified specific sequence(s) to be cloned obtained from step (f) into one or more of said cloning vectors in the presence of a ligase, said amplified sequence(s) and vector(s) being present in sufficient amounts to effect the ligation.

In a product embodiment, the invention provides a composition of matter useful in amplifying at least one specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, comprising four different nucleotide triphosphates and one oligonucleotide primer for each different specific sequence being amplified, wherein each primer is selected to be substantially complementary to different strands of each specific sequence, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer.

In another product embodiment, the invention provides a sample of one or more nucleic acids comprising multiple strands of a specific nucleic acid sequence contained in the nucleic acid(s). The sample may comprise about 10–100 of the strands, about 100–1000 of the strands, or over about 1000 of the strands.

In a final product embodiment, the invention provides an amplified nucleic acid sequence from a nucleic acid or mixture of nucleic acids comprising multiple copies of the sequence produced by the amplification processes herein.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. The oligonucleotide may be derived synthetically or by cloning.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleoside triphosphates and a thermostable enzyme in an appropriate buffer ("buffer" includes pH, ionic strength, cofactors, etc.) and at a suitable temperature. For Taq polymerase, the buffer herein preferably contains 1.5–2 mM of a magnesium salt, preferably $MgCl_{12}$, 150–200 μM of each nucleotide, and 1 μM of each primer, along with preferably 50 mM KCl, 10 mM Tris buffer at pH 88.4, and 100 μg/ml gelatin.

The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the thermostable enzyme. The exact lengths of the primers will depend on many factors, including temperature and source of primer and use of the method. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15–25 or more nucleotides, although it may contain more or fewer nucleotides. Short primer molecules generally require lower temperatures to form sufficiently stable hybrid complexes with the template.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5′ end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer. However, for detection purposes, particularly using labeled sequence-specific probes, the primers typically have exact complementarity to obtain the best results.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism" refers to the condition in which two or more different

nucleotide sequences can exist at a particular site in DNA.

As used herein, the term "nucleotide variation in sequence" refers to any single or multiple nucleotide substitutions, deletions or insertions. These nucleotide variations may be mutant or polymorphic allele variations. Therefore, the process herein can detect single nucleotide changes in nucleic acids such as occur in 8globin genetic diseases caused by single-base mutations, additions or deletions (some β-thalassemias, sickle cell anemia, hemoglobin C disease, etc.), as well as multiple-base variations such as are involved with β-thalassemia or some β-thalassemias. In addition, the process herein can detect polymorphisms, which are not necessarily associated with a disease, but are merely a condition in which two or more different nucleotide sequences (whether having substituted, deleted or inserted nucleotide base pairs) can exist at a particular site in the nucleic acid in the population, as with HLA regions of the human genome and random polymorphisms such as mitochondrial DNA. The polymorphic sequence-specific oligonucleotide probes described in detail hereinafter may be used to detect genetic markers linked to a disease such as insulin-dependent diabetis mellitus or in forensic applications. If the nucleic acid is double-stranded, the nucleotide variation in sequence becomes a base pair variation in sequence.

The term "sequence-specific oligonucleotides" refers to oligonucleotides which will hybridize to specific sequences whether or not contained on alleles, which sequences span the base pair variation being detected and are specific for the sequence variation being detected. Depending on the sequences being analyzed, one or more sequence-specific nucleotides may be employed for each sequence, as described further hereinbelow.

As used herein, the term "restriction fragment length polymorphism" ("RFLP") refers to the differences among individuals in the lengths of restriction fragments formed by digestion with a particular restriction endonuclease.

As used herein, the term "thermostable enzyme" refers to an enzyme which is stable to heat and is heat resistant and catalyzes (facilitates) combination of the nucleotides in the proper manner to form the primer extension products which are complementary, to each nucleic acid strand. Generally, the synthesis will be initiated at the 3′ end of each primer and will proceed in the 5′ direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be thermostable enzymes, however, which initiate synthesis at the 5′ end and proceed in the other direction, using the same process as described above.

The thermostable enzyme herein must satisfy a single criterion to be effective for the amplification reaction of this invention, i.e., the enzyme must not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. Irreversible denaturation for purposes herein refers to permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 90 to about 105° C. for a time depending mainly on the temperature and the nucleic acid length, typically about 0.5 to four minutes. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the nucleic acid is increased. Preferably, the enzyme will not become irreversibly denatured at about 90–100° C.

The thermostable enzyme herein preferably has an optimum temperature at which it functions which is higher than about 40° C., which is the temperature below which hybridization of primer to template is promoted, although, depending on (1) magnesium and salt concentrations and (2) composition and length of primer, hybridization can occur at higher temperatures (e.g., 45–70° C.). The higher the temperature optimum for the enzyme, the greater the specificity and/or selectivity of the primer-directed extension process. However, enzymes that are active below 40° C., e.g., at 37° C., are also within the scope of this invention, provided they are heat-stable. Preferably, the optimum temperature ranges from about 50 to 80° C, more preferably 60–80° C.

Examples of enzymes which have been reported in the literature as being resistant to heat include heat-stable polymerases, such as, e.g., polymerases extracted from the thermostable bacteria *Thermus flavus, Thermus ruber, Thermus thermophilus, Bacillus stearothermophilus* (which has a somewhat lower temperature optimum than the others listed), *Thermus aquaticus, Thermus lacteus, Thermus rubens,* and *Methanothermus fervidus.*

The preferred thermostable enzyme herein is a DNA polymerase isolated from *Thermus aquaticus,* strain YT-1, and purified as described in now abandoned U.S. patent application Ser. No. 899,241 filed Aug. 22, 1986, the disclosure of which is incorporated herein by reference. Briefly, *Thermus aquaticus* cells are grown and the polymerase is isolated and purified from the crude extract using the first five steps indicated by Kaledin et al., *Biokhimiya,* 45, 644–651 (1980), the disclosure of which is incorporated herein by reference. During the fifth step (DEAE column at pH 7.5), an assay is made for contaminating endo/exonucleases and only those fractions with polymerase activity and minimal nuclease contamination are pooled. The last chromatographic purification step uses a phosphocellulose column suggested by Chien et al., *J. Bacteriol.,* 127:1550–1557 (1976), the disclosure of which is incorporated herein by reference. Nuclease(s) and polymerase activities are assayed as described above, and only those polymerase fractions with minimal nuclease contamination are pooled.

While Kaledin et al. and Chien et al. report a purified enzyme with a molecular weight of 62–63 kdaltons, data using the modified purification protocol described above suggest a molecular weight of about 86–90 kdaltons.

The thermostable enzyme may be produced by recombinant DNA techniques by the method described U.S. Pat. No. 4,889,818, entitled "Purified Thermostable Enzyme", to Gelfand et al., the disclosure of which is incorporated herein by. reference. The thermostable enzyme also may be stored stably in a buffer as described in that same U.S. application.

The present invention is directed to a process for amplifying any one or more desired specific nucleic acid sequences contained in or suspected of being in a nucleic acid. Because large amounts of a specific sequence may be produced by this process, the present invention may be used for improving the efficiency of cloning DNA or messenger RNA and for amplifying a target sequence to facilitate detection thereof.

In general, the present process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given (a) that the ends of the specific sequence are known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and (b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any nucleic acid, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it contains or is suspected of containing the specific sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single stranded or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid.

It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA, or a portion of nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, organelles, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi or amniotic cells by a variety of techniques such as that described by Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982), 280–281.

For the process using sequence-specific probes to detect the amplified material, the cells may be directly used without purification of the nucleic acid if they are suspended in hypotonic buffer and heated to about 90–100° C., until cell lysis and dispersion of intracellular components occur, generally 1 to 15 minutes. After the heating step the amplification reagents may be added directly to the lysed cells.

Any specific nucleic acid sequence can be produced by the present process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid of defined length. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the efficiency of the process.

It will be understood that the word "primer" as used hereinafter may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified. For instance, in the case where a nucleic acid sequence is inferred from protein sequence information, a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the desired sequence to be amplified.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* (1981), 22:1859–1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. The first step involves contacting each nucleic acid strand with four different nucleoside triphosphates and one oligonucleotide primer for each different nucleic acid sequence being amplified or detected. If the nucleic acids to be amplified or detected are DNA, then the nucleoside triphosphates are dATP, dCTP, dGTP and TTP.

The nucleic acid strands are used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any. suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7–9, most preferably about 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6 1 primer:template) of the two oligonucleoside primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

Preferably, the concentration of nucleoside triphosphates is 150–200 μM each in the buffer for amplification, and MgCl is present in the buffer in an amount of 1.5–2 mM to increase the efficiency and specificity of the reaction.

The resulting solution is then treated according to whether the nucleic acids being amplified or detected are double or singlestranded. If the nucleic acids are

single-stranded, then no denaturation step need by employed, and the reaction mixture is held at a temperature which promotes hybridization of the primer to its complementary target (template) sequence. Such temperature is generally from about 35 to 65° C. or higher, preferably room 37–60° C., for an effective time, generally one-half to five minutes, preferably one-three minutes. Preferably, 45–58° C. is used for Taq polymerase and >15-mer primers to increase the specificity of primer hybridization. Shorter primers need lower temperatures.

The complement to the original single-stranded nucleic acid may be synthesized by adding one or two oligonucleoside primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, the thermostable enzyme and the nucleoside triphosphates. The product will be partially complementary, to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of strands of unequal length which may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added to the single-stranded nucleic acid and the reaction carried out.

If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One preferred physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 90 to 105° C for times generally ranging from about 0.5 to 5 minutes. Preferably the effective denaturing temperature is 90–100° C for 0.5 to 3 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Kuhn Hoffmann-Berling, *CSH-Quantitative Biology,* 43:63 (1978), and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics.,* 16:405–37 (1982). The denaturation produces two separated complementary strands of equal or unequal length.

If the double-stranded nucleic acid is denatured by heat, the reaction mixture is allowed to cool to a temperature which promotes hybridization of each primer present to its complementary. target (template) sequence. This temperature is usually from about 35 to about 65° C. or higher, depending on reagents, preferably from about 37° C. to about 60° C., maintained for an effective time, generally 0.5 to 5 minutes, and preferably 1–3 minutes. In practical terms, the temperature is simply lowered from about 95° C. to about 65° C. or to as low as 37° C., preferably to about 45–58° C. for Taq polymerase, and hybridization occurs at a temperature within this range.

Whether the nucleic acid is single- or double-stranded, the thermostable enzyme may be added at the denaturation step or when the temperature is being reduced to or is in the range for promoting hybridization. The reaction mixture is then heated to a temperature at which the activity of the enzyme is promoted or optimized, i.e., a temperature sufficient to increase the activity of the enzyme in facilitating synthesis of the primer extension products from the hybridized primer and template. The temperature must actually be sufficient to synthesize an extension product of each primer which is complementary to each nucleic acid template, but must not be so high as to denature each extension product from its complementary template (i.e., the temperature is generally less than about 80° C. 90° C.).

Depending mainly on the types of enzyme and nucleic acid(s) employed, the typical temperature effective for this synthesis reaction generally ranges from about 40 to 80° C., preferably 50–75° C. The temperature more preferably ranges from about 65–75° C. when a polymerase from *Thermus aquaticus* is employed. The period of time required for this synthesis may range from about 0.5 to 40 minutes or more, depending mainly on the temperature, the length of the nucleic acids, the enzyme and the complexity of the nucleic acid mixture, preferably one to three minutes. If the nucleic acid is longer, a longer period of time is generally required. The presence of dimethylsulfoxide (DMSO) is not necessary or recommended because DMSO was found to inhibit Taq polymerase enzyme activity.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands of the double-stranded molecule are separated by heat denaturation at a temperature effective to denature the molecule, but not so high that the thermostable enzyme is completely and irreversibly denatured or inactivated. Depending mainly on the type of enzyme and the length of nucleic acid, this temperature generally ranges from about 90 to 105° C., more preferably 90–100° C., and the time for denaturation typically ranges from 0.5 to four minutes, depending mainly on the temperature and the nucleic acid length.

After this time, the temperature is decreased to a level which promotes hybridization of the primer to its complementary single-stranded molecule (template) produced from the previous step. Such temperature is described above.

After this hybridization step, or in lieu of (or concurrently with) this hybridization step, the temperature is adjusted to a temperature which is effective to promote the activity of the thermostable enzyme to enable synthesis of a primer extension product using as template the newly synthesized strand from the previous step. The temperature again must not be so high as to separate (denature) the extension product from its template, as previously described (usually from 40 to 80° C. for 0.5 to 40 minutes, preferably 50 to 70° C. for 1–3 minutes). Hybridization may occur during this step, so that the previous step of cooling before denaturation is not required. In such a case, using simultaneous steps, the preferred temperature range is 50° C.–70° C.

The heating and cooling steps of strand separation, hybridization, and extension product synthesis can be repeated as often as needed to produce the desired quantity, of the specific nucleic acid sequence, depending on the ultimate use. The only limitation is the amount of the primers, the thermostable enzyme and the nucleoside triphosphates present. Preferably, the steps are repeated at least once. For use in detection, the number of cycles will depend, e.g., on the nature of the sample. For example, fewer cycles will be required if the sample being amplified is pure. If the sample is a complex mixture of nucleic acids, more cycles will be required to amplify, the signal sufficiently for its detec-

tion. For general amplification and detection, preferably the process is repeated at least 20 times.

When labeled sequence-specific probes are employed as described below, preferably the steps are repeated at least five times. When human genomic DNA is employed with such probes, the process is repeated preferably 15–30 times to amplify, the sequence sufficiently that a clearly detectable signal is produced, i.e., so that background noise does not interfere with detection.

As will be described in further detail below, the amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

No additional nucleosides, primers, or thermostable enzyme need be added after the initial addition, provided that the enzyme has not become denatured or inactivated irreversibly, in which case it is necessary to replenish the enzyme after each denaturing step. Addition of such materials at each step, however, will not adversely affect the reaction.

When it is desired to produce more than one specific nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of different oligonucleoside primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzymes in any known manner (e.g., by adding EDTA, CHC_{13}. SDS or phenol) or by separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an automated process, the reaction mixture may be temperature cycled such that the temperature is programmed to be controlled at a certain level for a certain time.

One such instrument for this purpose is the automated machine for handling the amplification reaction of this invention described in now abandoned U.S. patent application Ser. No. 833,368 filed Feb. 25, 1986 entitled "Apparatus And Method For Performing Automated Amplification of Nucleic Acid Sequences And Assays Using Heating And Cooling Steps," the disclosure of which is incorporated herein by reference. Briefly, this instrument utilizes a liquid handling system under computer control to make liquid transfers of enzyme stored at a controlled temperature in a first receptacle into a second receptacle whose temperature is controlled by the computer to conform to a certain incubation profile. The second receptacle stores the nucleic acid sequence(s) to be amplified plus the nucleoside triphosphates and primers. The computer includes a user interface through which a user can enter process parameters which control the characteristics of the various steps in the amplification sequence such as the times and temperatures of incubation, the amount of enzyme to transfer, etc.

A preferred machine which may be employed utilizes temperature cycling without a liquid handling system because the enzyme need not be transferred at every cycle. Such a machine is described more completely in copending U.S. patent application Ser. No. 899,061 filed Aug. 22, 1986, the disclosure of which is incorporated herein by reference. Briefly, this instrument consists of the following systems:

1. A heat-conducting container for holding a given number of tubes, preferably 500 μl tubes, which contain the reaction mixture of nucleoside triphosphates, primers, nucleic acid sequences, and enzyme.

2. A means to heat, cool, and maintain the heat-conducting container above and below room temperature, which means has an input for receiving a control signal for controlling which of the temperatures at or to which the container is heated, cooled or maintained. (This may be Peltier heat pumps available from Materials Electronics Products Corporation in Trenton, N.J. or a water heat exchanger.)

3. A computer means (e.g., a microprocessor controller), coupled to the input of said means, to generate the signals which control automatically the amplification sequence, the temperature levels, and the temperature ramping and timing.

In another embodiment, the enzyme used for the synthesis of primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series. Thus, the nucleic acids produced can be repeatedly denatured without inactivating the enzyme.

The present invention is demonstrated diagrammatically below where double-stranded DNA containing the desired sequence [S] comprised of complementary, strands [S+] and [S−] is utilized as the nucleic acid. During the first and each subsequent reaction cycle extension of each oligonucleoside primer on the original template will produce one new ssDNA molecule product of indefinite length which terminates with only one of the primers. These products, hereafter referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleoside primers during subsequent cycles and will produce molecules of the desired sequence [S+] or [S^{31}]. These molecules will also function as templates for one or the other of the oligonucleoside primers, producing further [S+] *and* [S−], and thus a chain reaction can be sustained which will result in the accumulation of [S] at an exponential rate relative to the number of cycles.

By-products formed by oligonucleoside hybridizations other than those intended are not self-catalytic (except in rare instances) and thus accumulate at a linear rate.

The specific sequence to be amplified, [S], can be depicted diagrammatically as:

[S+] 5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
[S−] 3′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′

The appropriate oligonuceotide primers would be:

Primer 1: GGGGGGGGGG
Primer 2: AAAAAAAAAA

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-continued

so that if DNA containing [S]

. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

is separated into single strands and its single strands are hybridized to Primers **1** and **2**, the following extension reactions can be catalyzed by a thermostable polymerase in the presence of the four nucleotide triphosphates:

3′ 5′
extends ← GGGGGGGGGG Primer 1
. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

original template strand−
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

Primer 2 AAAAAAAAAA → extends
5′ 3′

On denaturation of the duplexes formed, the products are:

3′ 5′
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG
newly synthesized long product 1

5′ 3′
. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

3′ 5′
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
original template strand−

5′ 3′
AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
newly synthesized long product 2

If these four strands are allowed to rehybridize with Primers **1** and **2** in the next cycle, the thermostable polymerase will catalyze the following reactions:

Primer 2 5′ AAAAAAAAAA → extends to here
3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly synthesized long product 1

extends ← GGGGGGGGGG 5′ Primer 1
5′ . . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
original template strand+

Primer 2 5′ AAAAAAAAAA → extends
3′ . . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzz . . . 5′
original template strand−

extends to here ← GGGGGGGGGG 5′ Primer 1
5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
newly synthesized long product 2

If the strands of the above four duplexes are separated, the following strands are found:

5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
newly synthesized [S+]

3′ . . . zzzzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
first cycle synthesized long product 1

3′ . . . zzzzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′
newly synthesized long product 1

5′ . . . zzzzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzz . . . 3′
original template strand+

5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . . 3′
newly synthesized long product 2

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-continued

3' . . . zzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzz . . . 5'
original template strand⁻

3' TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5'
newly synthesized [S⁻]

5' AAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzz . . . 3'
first cycle synthesized long product 2

It is seen that each strand which terminates with the oligonucleotide sequence of one primer and the complementary sequence of the other is the specific nucleic acid sequence [S] that is desired to be produced.

The amount of original nucleic acid remains constant in the entire process, because it is not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species. This is illustrated in the following table, which indicates the relative amounts of the species theoretically present after n cycles, assuming 100% efficiency at each cycle:

	Number of Double Strands After 0 to n Cycles		
Cycle Number	Template	Long Products	Specific Sequence [S]
0	1	—	—
1	1	1	0
2	1	2	1
3	1	3	4
5	1	5	26
10	1	10	1013
15	1	15	32,752
20	1	20	1,048,555
n	1	n	$(2^n$-n-1)

When a single-stranded nucleic acid is utilized as the template, only one long products is formed per cycle.

A sequence within a given sequence can be amplified after a given number of amplifications to obtain greater specificity of the reaction by adding after at least one cycle of amplification a set of primers which are complementary to internal sequences (which are not on the ends) of the sequence to be amplified. Such primers may be added at any stage and will provide a shorter amplified fragment. Alternatively, a longer fragment can be prepared by using primers with non-complementary ends but having some overlap with the primers previously utilized in the amplification.

The method herein may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard methods of recombinant DNA technology.

The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture if the original template DNA contains multiple target sequences, such as in a rheterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs produced by the amplification reaction. In one such modification, a restriction site is contained in each of the primers or in the sequence to be amplified and cloned. Preferably, the same or different restriction sites are incorporated at the 5' ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate enzymes, the amplified product can then be easily inserted into plasmid or viral vectors and cloned. This cloning allows the analysis or expression of individual amplified products, not a mixture.

If the primers have restriction sites incorporated therein, the same restriction site can be used for both primers. The use, however, of different sites allows the insertion of the product into the vector with a specific orientation and suppresses multiple insertions as well as insertions arising from amplifications based on only one of the two primers. The specific orientation is useful when cloning into single-strand sequencing vectors, when single-strand hybridization probes are used, or when the cloned product is being expressed.

One method to prepare the primers is to choose a primer sequence which differs minimally from the target sequence. Regions in which each of the primers is to be located are screened for homology to restriction sites appropriate to the desired vector. For example, the target sequence "CAGTATCCGA..." differs by only one base from one containing a BamHI site. A primer sequence is chosen to match the target exactly at its 3' end, and to contain the altered sequence and restriction site near its 5' end (for example, "CAGgATCCGA...", where the lower case letter symbolizes a mismatch with the target sequence). This minimally altered sequence will not interfere with the ability of the primer to hybridize to the original target sequence and to initiate polymerization. After the first amplification cycle the primer is copied, becomes the target, and matches exactly with new primers.

After the amplification process, the products are cleaved with the appropriate restriction enzymes, the restriction digest is optionally separated from inhibitors of ligation such as the nucleoside triphosphates and salts by, for example, passing over a desalting column, or a molecular weight chromatography column, or through a membrane, and the digestion product(s) containing the amplified sequence to be cloned is/are inserted by ligation into a cloning vector such as bacteriophage M13. The cloning vector generally has a selectable marker and may optionally also have a promoter. The gene may then be sequenced and/or expressed, if it codes for a protein, using well known techniques. The gene may also be sequenced by adding an appropriate primer during the amplification process which primer is complementary to the desired portion which is to be sequenced. The primer will form an extension product, and the extent of amplification with such extension product will provide sequence information.

Another method for preparing the primers involves taking the 3' end of the primers from the target se-

quence and adding the desired restriction site(s) to the 5' end of the primer. For the above example, a HindIII site could be added to make the sequence "cgaagcttCAGTATCCGA...", where lower case letters are as described above. The added bases would not contribute to the hybridization in the first cycle of amplification, but would match in subsequent cycles. The final amplified products are then cut with restriction enzyme(s) and cloned and expressed as described above. The gene being amplified may be, for example, human beta-hemoglobin or the human HLA DQ, DR or DP-α and -β genes.

In an alternative, but less preferred and less efficient, method of cloning wherein blunt-end ligation is employed rather than sticky-end ligation (using restriction enzymes), the basic amplification procedure is employed without concern for restriction enzymes in the primers or sequence(s) to be cloned. The steps must be repeated sufficiently, however, to produce enough amplified sequence(s) to effect ligation. Blunt-end ligation requires greater concentrations of sequence(s) and cloning vector(s) to be present than sticky-end ligation. In addition, the ligation must take place in the presence of a ligase, such as T4 ligase, *E. coli* ligase and ligase. Once the amplified product is obtained, the ligation procedure is a standard procedure using conditions well known to those skilled in the art.

The cloning method which does not involve blunt end ligation controls the orientation or multiplicity of insertion of the amplified product into the cloning vector.

In addition, the process herein can be used for in vitro mutagenesis. The oligonucleoside primers need not be exactly complementary to the nucleic acid sequence which is being amplified. It is only necessary that they be able to hybridize to the sequence sufficiently well to be extended by the thermostable enzyme. The product of an amplification reaction wherein the primers employed are not exactly complementarY to the original template will contain the sequence of the primer rather than the template, thereby introducing an in vitro mutation. In further cycles this mutation will be amplified with an undiminished efficiency because no further mispaired primings are required. The mutant thus produced may be inserted into an appropriate vector by standard molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers to induce further sequence changes. In this way a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last in a minor way., but from the original DNA source sequence in an increasingly major way. In this manner changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

In addition, the primer can contain as part of its sequence a non-complementary sequence, provided that a sufficient amount of the primer contains a sequence which is complementary to the strand to be amplified. For example, a nucleoside sequence which is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5' end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new template containing the non-complementary nucleoside insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

The method herein may also be used to enable detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirable.

For purposes of this invention genetic diseases may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, α-thalassemia, β-thalassemia, and the like. Sickle cell anemia can be readily detected via oligomer restriction analysis as described in EP Patent Publication No. 164,054 published Dec. 11, 1985, or via a RFLP-like analysis following amplification of the appropriate DNA sequence by the present method. u-Thalassemia can be detected by the absence of a sequence, and βthalassemia can be detected by the presence of a polymorphic restriction site closely linked to a mutation that causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA from, e.g., amniotic fluid containing a very low level of the desired sequence is amplified, cut with a restriction enzyme, and analyzed via a Southern blotting technique. The use of nonradioactive probes is facilitated by the high level of the amplified signal.

In another embodiment, a small sample of DNA may be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleoside derivatives which are readily detectable (such as ^{32}p-labeled or biotin-labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by restriction and electrophoretic separation or any other appropriate method.

In a further embodiment, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be detected by an appropriate method.

A practical application of this technique can be illustrated by its use in facilitating the detection of sickle cell anemia via the oligomer restriction technique described herein and in EP 164,054, *supra,* and Saiki et al., *Bio/-Technology,* 3, pp 1008–1012 (1985), the disclosures of which are incorporated herein by reference. Sickle cell anemia is a hemoglobin disease which is caused by a single base pair change in the sixth codon of the β-globin gene.

The method of this invention may also be used to detect directly single-base pair variations in nucleic acid sequence (such as genomic DNA) using sequence-specific oligonucleotides. This particular method is described in copending U.S. Ser. No. 899,344 filed Aug. 22, 1986, the disclosure of which is incorporated herein by reference.

In this method, the sequence variation, whether resulting from cancer, an infectious disease, or a genetic disease, e.g., a genetic lesion, is directly detected, eliminating the need for restriction digestion, electrophoresis, and gel manipulations otherwise required. The use of sequence-specific oligonucleotides in a dot blot format after amplification, as described herein, provides for improved specificity and sensitivity of the probe; an interpretable signal can be obtained with a 0.04 μg sample in six hours. Also, if the amount of sample spotted on a membrane is increased to 0.1–0.5 μg, non-isotopically labeled oligonucleotides may be utilized rather than the radioactive probes used in previous methods. Furthermore, the process described hereinbelow is applicable to use of sequence-specific oligonucleotides less than 19-mers in size, thus allowing use of more discriminatory sequence-specific oligonucleotides.

Regarding genetic diseases, while RFLP requires a polymorphic restriction site to be associated with the disease, sequence-specific oligonucleotides directly detect the genetic lesion and are generally more useful for the analysis of such diseases as hemoglobin C disease, α-1-antitrypsin and β-thalassemia, which result from single-base mutations. In addition, the oligonucleotides can be used to distinguish between genetic variants which represent different alleles (e.g., HLA typing), indicating the feasibility of a sequence-specific oligonucleoside-based HLA typing kit that includes a thermostable enzyme.

In one embodiment of the invention herein wherein a nucleoside variation in sequence is to be detected, the sample, amplified as described above using one primer for each strand of each nucleic acid suspected of containing the nucleoside variation, is spotted directly on a series of membranes and each membrane is hybridized with a different labeled sequence-specific oligonucleoside probe. One procedure for spotting the sample on a membrane is described by Kafotos et al., *Nucleic Acids Research,* 7:1541–1552 (1979), the disclosure of which is incorporated herein by reference.

Briefly, the DNA sample affixed to the membrane may be pretreated with a prehybridization solution containing sodium dodecyl sulfate, Ficoll, serum albumin and various salts prior to the probe being added. Then, a labeled oligonucleoside probe which is specific to each sequence variation to be detected is added to a hybridization solution similar to the prehybridization solution. The hybridization solution is applied to the membrane and the membrane is subjected to hybridization conditions that will depend on the probe type and length, the type and concentration of ingredients, etc. Generally, hybridization is carried out at about 25° to 75° C., preferably 35 to 65° C., for 0.25–50 hours, preferably less than three hours. The greater the stringency of conditions, the greater the required complementarity for hybridization between the probe and sample. If the background level is high, stringency may be increased accordingly. The stringent conditions can also be incorporated in the wash.

After the hybridization, the sample is washed of unhybridized probe using any suitable means such as by washing one or more times with varying concentrations of standard saline phosphate EDTA (SSPE) (180 mM NaCl, 10 mM $NaHPO_4$ and 1 M EDTA, pH 7.4) solutions at 25–75° C. for about 10 minutes to one hour, depending on the temperature. The label is then detected by using any appropriate detection technique.

The sequence-specific oligonucleoside employed herein is an oligonucleoside which is generally prepared and selected as described above for preparing and selecting the primers. As described above, the sequence-specific oligonucleoside must encompass the region of the sequence which spans the nucleoside variation being detected and must be specific for the nucleoside variation being detected. For example, if it is desired to detect whether a sample contains the mutation for sickle cell anemia, one oligonucleoside will be prepared which contains the nucleoside sequence site characteristic of the normal β-globin gene and one oligonucleoside will be prepared which contains the nucleoside sequence characteristic of the sickle cell allele. Each oligonucleoside would be hybridized to duplicates of the same sample to determine whether the sample contains the mutation.

The polymorphic areas of HLA class 11 genes are localized to specific regions of the first exon and are flanked by conserved sequences, so that oligonucleoside primers complementary to opposite strands of the conserved 5′ and 3′ ends of the first exon can be prepared.

The number of oligonucleotides employed for detection of the polymorphic areas of the HLA class 11 genes will vary depending on the type of gene, which has regions of base pair variation which may be clustered or spread apart. If the regions are clustered, as in the case with HLA-DQ-α, then one oligonucleoside is employed for each allele. If the regions are spread apart, as in the case with HLA-DQ-β and HLA-DR-β, then more than one probe, each encompassing an allelic variant, will be used for each allele.. In the case of HLA-DQ-β and HLA-DR-βthree probes are employed for the three regions of the locus where allelic variations may, occur. For detection of insulindependent diabetes mellitus (IDDM) four probes for the HLA-DRβ second exon are employed.

Haplotypes can be inferred from segregation analysis in families or, in some cases, by direct analysis of the individual DNA sample. Specific allelic combinations (haplotypes) of sequencespecific oligonucleoside reactivities can be identified in heterozygous cells by using restriction enzyme digestion of the genomic DNA prior to amplification.

For example, if in DQβ one finds three highly variable subregions A, B, and C. within a single amplified region, and if there are six different sequences at each region (A1–6, B1-6, C_1-6), then an individual could be typed in the DQβ locus by sequence-specific oligonucleoside probe analysis as containing A1, A2., B2, B3; C1, C4, with the possible haplotype combinations of AI, B2, CI; AI, B2, C4; A2, B2, CI; A2, B2, C4., AI, B3, CI, A1, B3, C4; AI, B2, C1; and A1, B2, C4.

If the genomic DNA is digested with a polymorphic restriction enzyme prior to amplification, and if the enzyme cuts both alleles between the primers, there is no reactivity with the sequencespecific probes due to lack of amplification, and the result is uninformative. If the enzyme cuts neither allele, the probe results with digested and undigested genomic DNA are the same and the result is uninformative. If the enzyme cuts only one allele, however, then one can infer both haplotypes

by comparing the probe reactivity patterns on digested and undigested DNA.

The haplotypes can be deduced by comparing sequence-specific oligonucleoside reactivities with uncut genomic DNA and genomic DNA cut with one or several enzymes known to be polymorphic and to recognize sites between the primers.

The length of the sequence-specific oligonucleoside will depend on many factors, including the particular target molecule being detected, the source of oligonucleoside, and the nucleoside composition. For purposes herein, the sequence-specific oligonucleoside typically contains 15–25 nucleosides, although it may contain more or fewer nucleosides. While oligonucleotides which are at least 19 mers in length may enhance specificity and/or sensitivity, probes which are less than 19 mers, e.g., 16-mers, may show more sequence-specific discrimination presumably because a single mismatch is more destabilizing. Because amplification increases specificity so that a longer length is less critical, and hybridization and washing temperatures can be lower for the same salt concentration, it is preferred to use oligonucleotides which are less than 19 mers in length.

Where the sample is first placed on the membrane and then detected with the oligonucleoside, the oligonucleoside must be labeled with a suitable label moiety, which may be detected by spectroscopic, photochemical, biochemical, immunochemical or chemical means. Immunochemical means include antibodies which are capable of forming a complex with the oligonucleoside under suitable conditions, and biochemical means include polypeptides or lectins capable of forming a complex with the oligonucleoside under the appropriate conditions. Examples include fluorescent dyes, electron-dense reagents, enzymes capable of depositing insoluble reaction products or being detected chromogenically, such as horseradish peroxidase, alkaline phosphatase, a radioactive label such as ^{32}P, or biotin. If biotin is employed, a spacer arm may be utilized to attach it to the oligonucleoside. Preferably, the label moiety is horseradish peroxidase.

Alternatively, in one "reverse" dot blot format, at least one of the primers and/or at least one of the four nucleoside triphosphates is labeled with a detectable label, so that the resulting amplified sequence is labeled. These labeled moieties may be present initially in the reaction mixture or added during a later cycle of the amplification to introduce the label to the amplification product. Then an unlabeled sequence-specific oligonucleoside capable of hybridizing with the amplified nucleic acid sequence, if the sequence variation(s) (whether normal or mutant) is/are present, is spotted on (affixed to) the membrane under prehybridization conditions as described above. The amplified sample is then added to the pretreated membrane under hybridization conditions as described above. Finally, detection means are used to determine if an amplified sequence in the nucleic acid sample has hybridized to the oligonucleoside affixed to the membrane. Hybridization will occur only if the membrane-bound sequence containing the variation is present in the amplification product, i.e., only if a sequence of the probe is complementary to a region of the amplified sequence.

In another version of the "reverse" dot blot format, the amplification is carried out without employing a label as with the "forward" dot blot format described above, and a labeled sequencespecific oligonucleoside probe capable of hybridizing with the amplified nucleic acid sequence containing the variation, if present, is spotted on (affixed to) the membrane under prehybridization conditions as described above. The amplified sample is then added to the pretreated membrane under hybridization conditions as described above. Then the labeled oligonucleoside or a fragment thereof is released from the membrane in such a way that a detection means can be used to determine if an amplified sequence in the sample hybridized to the labeled oligonucleoside. The release may take place, for example, by adding a restriction enzyme to the membrane which recognizes a restriction site in the probe. This procedure, known as oligomer restriction, is described more fully in EP Patent Publication 164,054 published Dec. 11, 1985, the disclosure of which is incorporated herein by reference.

In both the forward and reverse dot blot methods, the genetic diseases which may be detected include specific deletions, insertions and/or substitutions in any base pair mutation or polymorphism in nucleic acids, for example, genomic DNA, from any organism. Examples of diseases in which base pair variation is known include sickle cell anemia, hemoglobin C disease, α-thalassemia, β-thalassemia, and the like. Other diseases that may be detected include cancerous diseases such as those involving the RAS oncogenes, e.g., the n-RAS oncogene, and infectious diseases.

A dot blot process may also be used for HLA typing in the areas of tissue transplantation, disease susceptibility, and paternity determination. The HLA class 11 genes, consisting of the α0 and β 9enes from the HLA-DR, HLA-DQ and HLA-DP regions, are highly polymorphic; their genetic complexity at the DNA level is significantly greater than the polymorphism currently defined by serological typing. In addition, the process herein may be employed to detect certain DNA sequences coding for HLA class 11 proteins (e.g., DRB) associated with insulin-dependent diabetes melliters, (IDDM) as described more fully in copending U.S. Ser. No 899,512 filed Aug. 22, 1986, the disclosure of which is incorporated herein by reference. Briefly, the four DNA sequences associated with IDDM are selected from the group consisting of:

(1) 5'-GAGCTGCGTAAGTCTGAG-3',
(2) 5'-GAGGAGTTCCTGCGCTTC-3',
(3) 5'-CCTGTCGCCGAGTCCTGG-3', and
(4) 5'-GACATCCTGGAAGACGAGAGA-3', or the DNA strands that are complementary thereto. Sequence-specific probes may be prepared that will hybridize to one or more of these sequences.

Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism. These include bacteria, such as Salmonella, Chlamydia, Neisseria., viruses, such as the hepatitis viruses, and parasites, such as the Plasmodium responsible for malaria. U.S. Patent Reexamination Certificate B14,358,535 issued on May 13, 1986 to Falkow et al. describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these may constitute only a very small fraction of the total DNA in the sample. Specific amplification of suspected sequences prior to immobilization and hybridization detection of the DNA samples could greatly improve the sensitivity and specificity of traditional procedures.

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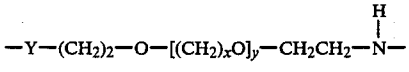
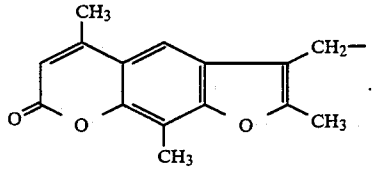
Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if nonradioactively labeled probes could be employed as described in EP 3,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of specific DNA amplification by the present method and the use of stably labeled probes could provide the convenience and sensitivity required to make the Falkow and Ward procedures useful in a routine clinical setting.

A specific use of the amplification technology for detecting or monitoring for the AIDS virus is described in copending U.S. Ser. No. 818,127 filed Jan. 10, 1986, the disclosure of which is incorporated herein by reference. Briefly, the amplification and detection process is used with primers and probes which are designed to amplify and detect, respectively, nucleic acid sequences which are substantially conserved among the nucleic acids in AIDS viruses and specific to the nucleic acids in AIDS viruses. Thus, the sequence to be detected must be sufficiently complementary to the nucleic acids in AIDS viruses to initiate polymerization, preferably at room temperature, in the presence of the enzyme and nucleoside triphosphates.

In addition, the probe may be a biotinylated probe in which the biotin is attached to a spacer arm of the formula:



where Y is O, NH or N–CHO, x is a number from 1 to 4, and y is a number from 2 to 4. The spacer arm is in turn attached to a psoralen moiety of the formula:



The psoralen moiety intercalates into and crosslinks a "gapped circle" probe as described by Courage-Tebbe et al., Biochim. Biophys. Acta, 697 (1982) 1–5, wherein the single-stranded hybridization region of the gapped circle spans the region contained in the primers. The details of this biotinylation and dot blot procedure are described more fully in commonly assigned U.S. Pat. No. 4,582,789 issued Apr. 5, 1986 and U.S. Pat. No. 4,617,261 issued Oct. 14, 1986, the disclosures of which are incorporated herein by reference.

The amplification process can also be utilized to produce sufficient quantities of DNA from a single copy human gene such that detection by a simple non-specific DNA stain such as ethidium bromide can be employed to diagnose DNA directly.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphism that may not be associated with any pathological state.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In these examples all percentages are by weight if for solids and by volume if for liquids, unless otherwise noted, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE I

I. Synthesis of the Primers

The following two oligonucleoside primers were prepared by the method described below:

5′-ACACAACTGTGTTCACTAGC-3′ (PC03)
5′-CAACTTCATCCACGTTCACC-3′ (PC04)

These primers, both 20-mers, anneal to opposite strands of the genomic DNA with their 5′ ends separated by a distance of 110 base pairs.

A. Automated Synthesis Procedures: The diethylphosphoramidites, synthesized according to Beaucage and Caruthers (*Tetrahedron Letters* (1981) 22:1859–1862) were sequentially condensed to a nucleoside derivatized controlled pore glass support using a Biosearch SAM-1. The procedure included detritylation with trichloroacetic acid in dichloromethane, condensation using benzotriazole as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine. Cycle time was approximately 30 minutes. Yields at each step were essentially quantitative and were determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

B. Oligodeoxyribonucleoside Deprotection and Purification Procedures: The solid support was removed from the column and exposed to 1 ml concentrated ammonium hydroxide at room temperature for four hours in a closed tube. The support was then removed by filtration and the solution containing the partially protected oligodeoxynucleoside was brought to 55° C for five hours. Ammonia was removed and the residue was applied to a preparative polyacrylamide gel. Electrophoresis was carried out at 30 volts/cm for 90 minutes after which the band containing the product was identified by UV shadowing of a fluorescent plate. The band was excised and eluted with 1 ml distilled water overnight at 4° C. This solution was applied to an Altech RP18 column and eluted with a 7–13% gradient of acetonitrile in 1% ammonium acetate buffer at pH 6.0. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

C. Characterization of Oligodeoxyribonucleosides: Test aliquots of the purified oligonucleotides were ^{32}P labeled with polynucleoside kinase and γ-^{32}P-ATP. The labeled compounds were examined by autoradiography of 14–20% polyacrylamide gels after electrophoresis for 45 minutes at 50 volts/cm. This procedure verifies the molecular weight. Base composition was determined by digestion of the oligodeoxyribonucleoside to nucleosides by use of venom diesterase and bacterial alkaline phosphatase and subsequent separation and quantitation of the derived nucleosides using a reverse phase HPLC column and a 10% acetonitrile, 1% ammonium acetate mobile phase.

II. Isolation of Human Genomic DNA from Cell Line

High molecular weight genomic DNA was isolated from a T cell line, Molt 4, homozygous for normal β-globin available from the Human Genetic Mutant Cell Depository, Camden, NJ as GM2219C using essentially the method of Maniatis et al., supra, p. 280–281.

III. Purification of a Polymerase From *Thermus aquaticus*

Thermus aquaticus strain YT1, available without restriction from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, as ATCC No. 25,104 was grown in flasks in the following medium:

Sodium Citrate	1 mM
Potassium Phosphate, pH 7.9	5 mM
Ammonium Chloride	10 mM
Magnesium Sulfate	0.2 mM
Calcium Chloride	0.1 mM
Sodium Chloride	1 g/l
Yeast Extract	1 g/l
Tryptone	1 g/l
Glucose	2 g/l
Ferrous Sulfate	0.01 mM

(The pH was adjusted to 8.0 prior to autoclaving.)

A 10-liter fermentor was inoculated from a seed flask cultured overnight in the above medium at 70° C. A total of 600 ml from the seed flask was used to inoculate 10 liters of the same medium. The pH was controlled at 8.0 with ammonium hydroxide with the dissolved oxygen at 40%, with the temperature at 70° C., and with the stirring rate at 400 rpm.

After growth of the cells, they were purified using the protocol (with slight modification) of Kaledin et al., supra, through the first five stages and using a different protocol for the sixth stage. All six steps were conducted at 4° C. The rate of fractionation on columns was 0.5 columns/hour and the volumes of gradients during elution were 10 column volumes. An alternative and preferred purification protocol is described in Gelfand et al., U.S. Pat. No. 4,889,818, entitled "Purified Thermostable Enzyme", the entire disclosure of which is incorporated herein by reference.

Briefly, the above culture of the T. aquaticus cells was harvested by centrifugation after nine hours of cultivation, in late log phase, at a cell density of 1.4 g dry weight/1. Twenty grams of cells were resuspended in 80 ml of a buffer consisting of 50 mM Tris.HCl pH 7.5, 0.1 mM EDTA. Cells were lysed and the lysate was centrifuged for two hours at 35,000 rpm in a Beckman TI 45 rotor at 4° C. The supernatant was collected (fraction A) and the protein fraction precipitating between 45 and 75% saturation of ammonium sulfate was collected, dissolved in a buffer consisting of 0.2 M potassium phosphate buffer, pH 6.5, 10 mM 2-mercaptoethanol, and 5% glycerine, and finally dialyzed against the same buffer to yield fraction B.

Fraction B was applied to a 2.2×30-cm column of DEAEcellulose, equilibrated with the above described buffer. The column was then washed with the same buffer and the fractions containing protein (determined by absorbance at 280 nm) were collected. The combined protein fraction was dialyzed against a second buffer, containing 0.01 M potassium phosphate buffer, pH 7.5, 10 mM 2mercaptoethanol, and 5% glycerine, to yield fraction C.

Fraction C was applied to a 2.6×21-cm column of hydroxyapatite, equilibrated with a second buffer. The column was then washed and the enzyme was eluted with a linear gradient of 0.01–0.5 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 5% glycerine. Fractions containing DNA polymerase activity. (90–180 mM potassium phosphate) were combined, concentrated four-fold using an Amicon stirred cell and YM10 membrane, and dialyzed against the second buffer to yield fraction D.

Fraction D was applied to a 1.6×28-cm column of DEAEcellulose, equilibrated with the second buffer. The column was washed and the polymerase was eluted with a linear gradient of 0.01–0.5 M potassium phosphate in the second buffer. The fractions were assayed for contaminating endonuclease(s) and exonuclease(s) by electrophoretically detecting the change in molecular weight of phage λ DNA or supercoiled plasmid DNA after incubation with an excess of DNA polymerase (for endonuclease) and after treatment with a restriction enzyme that cleaves the DNA into several fragments (for exonuclease). Only those DNA polymerase fractions (65–95 mM potassium phosphate) having minimal nuclease contamination were pooled. To the pool was added autoclaved gelatin in an amount of 250 μg/ml, and dialysis was conducted against the second buffer to yield Fraction E.

Fraction E was applied to a phosphocellulose column and eluted with a 100 ml gradient (0.01–0.4 M KCl gradient in 20 mM potassium phosphate buffer pH 7.5). The fractions were assayed for contaminating endo/exonuclease(s) as described above as well as for polymerase activity (by the method of Kaledin et al.) and then pooled. The pooled fractions were dialyzed against the second buffer, then concentrated by dialysis against 50% glycerine and the second buffer.

The molecular weight of the polymerase was determined by SDS PAGE. Marker proteins (Bio-Rad low molecular weight standards) were phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Preliminary data suggest that the polymerase has a molecular weight of about 86,000–90,000 daltons, not 62,000–63,000 daltons reported in the literature (e.g., by Kaledin et al.).

The polymerase was incubated in 50 μl of a mixture containing 25 mM Tris-HCl pH 6.4 and pH 8.0, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 nmoles each of dGTP, dATP, and TTP, and 0.5 μCi (^3H) dCTP, 8 μg "activated" calf thymus DNA, and 0.5–5 units of the polymerase. "Activated" DNA is a native preparation of DNA after partial hydrolysis with DNase I until 5% of the DNA was transferred to the acid-soluble fraction. The reaction was conducted at 70° C. for 30 minutes, and stopped by adding 50 ul of a saturated aqueous solution of sodium pyrophosphate containing 0.125 M EDTA-Na_2 Samples were processed and activity was determined as described by Kaledin et al., supra.

The results showed that at pH 6.4 the polymerase was more than one-half as active as at pH 8.0. In contrast, Kaledin et al. found that at pH about 7.0, the enzyme therein had 8% of the activity at pH 8.3. Therefore, the pH profile for the instant thermostable enzyme is broader than that for the Kaledin et al. enzyme.

Finally, when only one or more nucleoside triphosphates were eliminated from a DNA polymerase assay

reaction mixture, very little, if any, activity was observed using the enzyme herein, which activity was consistent with the expected value and with an enzyme exhibiting high fidelity. In contrast, the activity obtained using the Kaledin et al. (supra) enzyme is not consistent with the expected value, and suggests misincorporation of nucleoside triphosphate(s).

IV. Amplification Reaction

One microgram of the genomic DNA described above was diluted in an initial 100 μl aqueous reaction volume containing 25 mM Tris.HCl buffer (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 200 μg/ml gelatin, 1 μM of primer PC03, 1 μM of primer PC_{04}, 1.5 mM dATP, 1.5 mM dCTP, 1.5 mM dGTP and 1.5 mM TTP. The sample was heated for 10 minutes at 98° C. to denature the genomic DNA, then cooled to room temperature. Four microliters of the polymerase from *Thermus aquaticus* was added to the reaction mixture and overlaid with a 100 μl mineral oil cap. The sample was then placed in the aluminum heating block of the liquid handling and heating instrument described in copending U.S. Application Ser. No. 833,368 filed Feb. 25, 1986, the disclosure of which is incorporated herein by reference.

The DNA sample underwent 20 cycles of amplification in the machine, repeating the following program cycle:

(1) heating from 37° C. to 98° C. in heating block over a period of 2.5 minutes., and

(2) cooling from 98° C. to 37° C. over a period of three minutes to allow the primers and DNA to anneal.

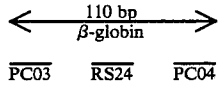
After the last cycle, the sample was incubated for an additional 10 minutes at 55° C. to complete the final extension

V. Synthesis and Phosphorylation of Oligodeoxyribonucleoside Probes

A labeled DNA probe, designated RS_{24}, of the following sequence was prepared:

5'*CCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAG-3' (RS24)

where * indicates the label. This probe is 40 bases long, spans the fourth through seventeenth codons of the gene, and is complementary to the normal β-globin allele (β^A). The schematic diagram of primers and probes is given below:



This probe was synthesized according to the procedures described in Section I of Example I. The probe was labeled by contacting 20 pmole thereof with 4 units of T4 polynucleoside kinase (New England Biolabs) and about 40 pmole γ-^{32}P-ATP (New England Nuclear, about 7000 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM spermine, and 10 mM dithiothreitol for 60 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., Molecular Cloning (1982), 466–467 over a ml Bio Gel P-4 (BioRad) spin dialysis column equilibrated with Tris-EDTA (TE) buffer (10 mM Tris buffer, 0.1 mM EDTA, pH 8.0). TCA precipitation of the reaction product indicated that for RS24 the Specific activity was 4.3 vCi/pmole and the final concentration was 0.118 pmole/μl.

VI. Dot Blot Hybridizations

Four microliters of the amplified sample from Section IV and 5.6 μl of appropriate dilutions of β-globin plasmid DNA calculated to represent amplification efficiencies of 70, 75, 80, 85, 90, 95 and 100% were diluted with 200 μl 0.4 N NaOH, 25 mM EDTA and spotted onto a Genatran 45 (Plasco) nylon filter by first wetting the filter with water, placing it in a Bio-Dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots which holds the filters in place, applying the samples, and rinsing each well with 0.1 ml of 20×SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM EDTA), as disclosed by Reed and Mann, *Nucleic Acids Research,* 13, 7202–7221 (1985). The filters were then removed, rinsed in 20×SSPE, and baked for 30 minutes at 80° C. in a vacuum oven.

After baking, each filter was then contacted with 16 ml of a hybridization solution consisting of 3×SSPE, 5×Denhardt's solution (1×=0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin,, 0.2 mM Tris, 0.2 mM EDTA, pH 8.0), 0.5% SDS and 30% formamide, and incubated for two hours at 42° C. Then 2 pmole of probe RS_{24} was added to the hybridization solution and the filter was incubated for two minutes at 42° C.

Finally, each hybridized filter was washed twice with 100 ml of 2×SSPE and 0.1% SDS for 10 minutes at room temperature. Then the filters were treated once with 100 ml of 2×SSPE, 0.1% SDS at 60° C. for 10 minutes.

Each filter was then autoradiographed, with the signal readily apparent after two hours.

VII. Discussion of Autoradiogram

The autoradiogram of the dot blots was analyzed after two hours and compared in intensity to standard serial dilution β-globin reconstructions prepared with HaeIII/MaeI-digested pBR.βA, where βA is the wild-type allele, as described in Saiki et al., *Science. supra.* Analysis of the reaction product indicated that the overall amplification efficiency was about 95%, corresponding to a 630,000-fold increase in the β-globin target sequence.

EXAMPLE II 1. Amplification Reaction

Two 1 μg samples of genomic DNA extracted from the Molt 4 cell line as described in Example I were each diluted in a 100 μl reaction volume containing 50 mM KCl, 25 mM Tris.HCl buffer pH 8.0, 10 mM $MgCl_{12}$, 1 μM of primer PCO_3 1 nM of primer PCO_4, 200 μg/ml gelatin, 10% dimethylsulfoxide (by volume), and 1.5 mM each of dATP, dCTP, dGTP and TTP. After this mixture was heated for 10 minutes at 98° C. to denature the genomic DNA, the samples were cooled to room temperature and 4 μl of the polymerase from *Thermus aquaticus* described in Example I was added to each sample. The samples were overlaid with mineral oil to prevent condensation and evaporative loss.

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One of the samples was placed in the heating block of the machine described in Example I and subjected to 25 cycles of amplification, repeating the following program cycle:

(1) heating from 37 to 93° C. over a period of 2.5 minutes.,

(2) cooling from 93° C. to 37° C. over a period of three minutes to allow the primers and DNA to anneal., and

(3) maintaining at 37° C. for two minutes.

After the last cycle the sample was incubated for an additional 10 minutes at 60° C. to complete the final extension reaction.

The second sample was placed in the heat-conducting container of the machine, described in more detail in copending U.S. patent application Ser. No. 899,061 filed Aug. 22, 1986. The heat-conducting container is attached to Peltier heat pumps which adjust the temperature upwards or downwards and a microprocessor controller to control automatically the amplification sequence, the temperature levels, the temperature ramping and the timing of the temperature. The second sample was subjected to 25 cycles of amplification, repeating the following program cycle: (1) heating from 37 to 95° C. over a period of three minutes; (2) maintaining at 95° C. for 0.5 minutes to allow denaturation to occur; (3) cooling from 95 to 37° C. over a period of one minute; (4) maintaining at 37° C. for one minute.

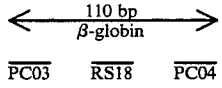
II. Analysis

Two tests were done for analysis, a dot blot and an agarose gel analysis.

For the dot blot analysis, a labeled DNA probe, designated RS_{18}, of the following sequence was prepared.

5'-*CTCCTGAGGAGAAGTCTGC-3'(RS18)

where * indicates the label. This probe is 19 bases long, spans the fourth through seventeenth codons of the gene, and is complementary to the normal μ-globin allele (μA). The schematic diagram of primers and probes is given below:



This probe was synthesized according to the procedures described in Section I of Example 1. The probe was labeled by contacting 10 pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and about 40 pmole $\gamma-^{32}$p-ATP (New England Nuclear, about 7000 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris.HCl buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM spermine and 10 mM dithiothreitol for 60 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., supra, p. 466–467 over a 1 ml Bio Gel P-4 (BioRad) spin dialysis column equilibrated with Tris-EDTA (TE) buffer (10 mM Tris.HCl buffer, 0.1 mM EDTA, pH 8.0). TCA precipitation of the reaction product indicated that for RS_{18} the specific activity was 4.6 μCi/pmole and the final concentration was 0.114 pmole/μl.

Five microliters of the amplified sample from Section I and of a sample amplified as described above except using the Klenow fragment of *E. coli* DNA Polymerase I instead of the thermostable enzyme were diluted with 195 μl 0.4 N NaOH, 25 mM EDTA and spotted onto two replicate Genatran 45 (Plasco) nylon filters by first wetting the filters with water, placing them in a Bio-Dot (Bio-Rad, Richmond, CA) apparatus for preparing dot blots which holds the filters in place, applying the samples, and rinsing each well with 0.4 ml of 20×SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM and Mann, supra. The filters were then removed, rinsed in 20×SSPE, and baked for 30 minutes at 80° C. in a vacuum oven.

After baking, each filter was then contacted with 6 ml of a hybridization solution consisting of 5×SSPE, 5×Denhardt's solution (1×=0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 0.2 mM Tris, 0.2 mM EDTA, pH 8.0) and 0.5% SDS, and incubated for 60 minutes at 55° C. Then 5 μl of probe RS18 was added to the hybridization solution and the filter was incubated for 60 minutes at 55° C.

Finally, each hybridized filter was washed twice with 100 ml of 2×SSPE and 0.1% SDS for 10 minutes at room temperature. Then the filters were treated twice more with 100 ml of 5×SSPE, 0.1% SDS at 60° C. for 1) one minute and 2) three minutes, respectively.

Each filter was then autoradiographed, with the signal readily apparent after 90 minutes.

In the agarose gel analysis, 5 μl each amplification reaction was loaded onto 4% NuSieve/0.5% agarose gel in 1×TBE buffer (0.089 M Tris, 0.089 M boric acid, and 2 mM EDTA) and electrophoresed for 60 minutes at 100 V. After staining with ethidium bromide, DNA was visualized by UV fluorescence.

The results show that the machines used in Example I and this example were equally effective in amplifying the DNA, showing discrete high-intensity 110-base pair bands of similar intensity, corresponding to the desired sequence, as well as a few other discrete bands of much lower intensity. In contrast, the amplification method as described in Example I of copending U.S. patent application Ser. No. 839,331 filed Mar. 13, 1986, supra, which involves reagent transfer after each cycle using the Klenow fragment of *E. coli* Polymerase I, gave a DNA smear resulting from the non-specific amplification of many unrelated DNA sequences.

It is expected that similar improvements in amplification and detection would be achieved in evaluating HLA-DQ, DR and DP regions.

If in the above experiments the amplification reaction buffer contains 2 mM $MgCl_2$ instead of 10 mM $MgCl_2$ and 150–200 μM of Z' and if the lower each nucleotide rather than 1.5 mM of each, temperature of 37° C. is raised to 45–58° C. during amplification, better specificity and efficiency of amplification occurs. Also, DMSO was found not necessary or preferred for amplification.

EXAMPLE III

Amplification and Cloning

For amplification of a 119-base pair fragment on the human μ-globin gene, a total of 1 microgram each of human genomic DNA isolated from the Molt 4 cell line or from the GM2064 cell line (representing a homozygous deletion of the β- and δ-hemoglobin region and available from the Human Genetic Mutant Cell Depository., Camden, NJ) as described above was amplified in a 100 μl reaction volume containing 50 mM KCl, 25 mM Tris.HCl pH 8, 10 mM $MgCl_2$, 200 μg/ml gelatin, 5

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mM 2-mercaptoethanol, 1.5 mM each of dATP, dCTP, TTP, and dGTP, and 1 μM of each of the following primers:

5′-CTTCTGcagCAACTGTGTTCACTAGC-3′ (GH18)
5′-CACaAgCTTCATCCACGTTCACC-3′ (GH19)

where lower case letters denote mismatches from wild-type sequence to create restriction enzyme sites. GH18 is a 26-base oligonucleotide complementary to the negative strand and contains an internal PstI site. GH19 is a 29-base oligonucleotide complementary to the plus strand and contains an internal HindIII recognition sequence. These primers were selected by first screening the regions of the gene for homology to the PstI and HindIII restriction sites. The primers were then prepared as described in Example I.

The above reaction mixtures were heated for 10 minutes at 5° C. and then cooled to room temperature. A total of 4 nl of the polymerase described in Example I was added to each reaction mixture, and then each mixture was overlayed with mineral oil. The reaction mixtures were subjected to 30 cycles of amplification with the following program:

2.5 min. ramp, 37 to 98° C.
3 min. ramp, 98 to 37° C.
2 min. soak, 37° C.

After the last cycle, the reaction mixtures were incubated for 20 minutes at 65° C. to complete the final extension. The mineral oil was extracted with chloroform and the mixtures were stored at −20° C.

A total of 10 μl of the amplified product was digested with 0.5 μg M13mp10 cloning vector, which is publicly available from Boehringer-Mannheim, in a 50 μl volume containing 50 mM NaCl, 10 mM Tris.HCl, pH 7.8, 10 mM $MgCl_{2,\ 20}$ units PstI and 26 units HindIII for 90 minutes at 37° C. The reaction was stopped by freezing at −20° C. The volume was adjusted to 110 μl with TE buffer and loaded (100 μl) onto a 1 ml BioGel P-4 spin dialysis column. One 0.1 ml fraction was collected and ethanol precipitated.

(At this point it was discovered that there was μ-globin amplification product in the GM2064 sample. Subsequent experiments traced the source of contamination to the primers, either GH18 or GH19. Because no other source of primers was available, the experiment was continued with the understanding that some cloned sequences would be derived from the contaminating DNA in the primers.)

The ethanol pellet was resuspended in 15 μl water, then adjusted to 20 μl volume containing 50 mM Tris.HCl, pH 7.8, 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM dithiothreitol, and 400 units ligase. [One unit is the amount of enzyme required to give 50% ligation of HindIII digested XDNA in 30 minutes at 16° C. in 20 μlat a 5′ termini concentration of 0.12 mM (about 330 μg/ml)]. This mixture was incubated for three hours at 16° C.

Ten microliters of ligation reaction mixture containing Molt digested λDNA was transformed into *E. coli* strain JMI03 competent cells, which are publicly available from BRL in Bethesda, MD. The procedure followed for preparing the transformed strain is described in Messing, J. (1981) Third Cleveland Symposium on Macromolecules:Recombinant DNA, ed. A. Walton, Elsevier, Amsterdam, 143–163. A total of 651 colorless plaques (and 0 blue plaques) were obtained. Of these, 119 had a (+)strand insert (18%) and 19 had a (−)-strand insert (3%). This is an increase of almost 20-fold over the percentage of μ-globin positive plaques among the primer-positive plaques from the amplification technique using Klenow fragment of *E. coli* Polymerase I, where the reaction proceeded for two minutes at 25° C., after which the steps of heating to 100° C. for two minutes, cooling, adding Klenow fragment, and reacting were repeated nine times. These results confirm the improved specificity of the amplification reaction employing the thermostable enzyme herein.

In a later cloning experiment with GM2064 and the contaminated primers, 43 out of 510 colorless plaques (8%) had the (+)- strand insert. This suggests that approximately one-half of the 119 clones from Molt 4 contain the contaminant sequence.

Ten of the (+)- strand clones from Molt 4 were sequenced. Five were normal wild-type sequence and five had a single C to T mutation in the third position of the second codon of the gene (CAC to CAT). Four of the contaminant clones from GM2064 were sequenced and all four were normal.

Restriction site-modified primers may also be used to amplify and clone and partially sequence the human N-ras oncogene and to clone segments of the HLA DQ-α, DQ-β and DR-β genes using the above technique.

Again, if the concentrations of $MgCl_2$ and nucleotides are reduced to 2 mM and 150–200 μM, respectively, and the minimum cycling temperature is increased from 37° C. to 45–58° C., the specificity and efficiency of the amplification reaction can be increased.

EXAMPLE IV

cDNA was made from 1 μg of rabbit reticulocyte mRNA (Bethesda Research Laboratories) in a 100 μl reaction volume containing 150 mM KC1, 50 mM Tris.HCI (pH 8.3), 10 mM $MgCl_{2,\ 5}$ mM DTT, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM TTP, 0.5 mM dGTP, 0.2 μg oligo(dT)1218 (Pharmacia), 40 units RNasin (Promega Biotec), and 5 units AMV reverse transcriptase (BRL) and incubated for 30 minutes at 42° C. The reaction was stopped by heating for 10 minutes at 95° C. Two μg RNase A was added to the sample (2 μl of a 2 mg/ml solution in water) and incubated for 10 minutes at 37° C.

Three amplification reactions were done with the Klenow fragment using different pairs of primers. The primer pair PCO3/PCO4 define a 110-bp product. The primer pair RS_{45}/oligo(dT)25–30 define an about 370-bp product, and the primer pair PCO3/oligo(dT)25–30 an about 600-bp product. PCO_3 PCO4, and RS_{45} are complementary to the human μ-globin gene and each has two mismatches with the rabbit gene. PCO3 and PCO4 are described in Example I. RS_{45} has the sequence: 5"-CAAGAAGGTGCTAGGTGCC-3".

The amplification reactions were performed with 1/20th (5 μl of the cDNA described above in a 100 μl reaction volume containing 50 mM NaCl, 10 mM Tris.HCl (pH 7.6), 10 mM $MgCl_{2,\ 200}$ μg/ml gelatin, 10% DMSO, 1 μM PCO3 or $RS_{45,\ 1}$ μM PCO4 or oligo(dT)25–30, 1.5 mM dATP, 1.5 mM dCTP, 1.5 mM TTP and 1.5 mM dGTP. The samples were heated for five minutes at 98° C., then cooled to room temperature and overlayed with 100 μl mineral oil.

The samples were subjected to 10 cycles of automated amplification using the machine described in Example I and using the following program:

(1) heating from 37° C. to 98° C. in a heating block over 2.5 minutes (denature);

(2) cooling from 98° C. to 37° C. over 3.0 minutes (anneal);

(3) adding 1 unit Klenow fragment, and

(4) maintaining at 37° C. for 20 minutes (extend).

The final volume of each sample was about 140 μl.

One-twentieth (7 μl) of each sample was analyzed by electrophoresis on a 2% agarose gel. After staining with ethidium bromide, discrete bands were seen in the PCO3/PCO4 and RS_{45}/oligo(dT) samples. The sizes of the bands were consistent with the expected lengths: 110-bp for the former, about 370-bp for the latter. No evidence of amplification of an about 600-bp fragment with the PCO3/oligo(dT) primer pair was observed.

The contents of the gel were Southern blotted onto a Genatran nylon membrane and hybridized with a nick-translated human 8globin probe, pBR328:betaA, described in Saiki et al., Science, supra, using standard techniques. The resulting autoradiogram extended the conclusions reached previously - the 110 and about 370-bp fragments were μ-globin specific amplification products and no significant amplification of the about 600-bp band was detected.

Three additional samples were amplified with the Taq polymerase obtained as described above using the same primer pairs described previously. Five microliter portions of cDNA were amplified in 100 μl reaction volumes containing 50 mM KCl, 25 mM Tris.HCl (pH 8.0), 10 mM $MgCl_{2,\ 200}$ μg/ml gelatin, 10% DMSO, 1 μM PCO3 or $RS_{45,\ 1}$ uM PCO4 or oligo-(dT)25–30, 1.5 mM dATP, 1.5 mM dCTP, 1.5 mM TTP and 1.5 mM dGTP. The samples were heated for five minutes at 98° C., then cooled to room temperature. One microliter of Taq polymerase (1/8 dilution of lot 2) was added to each and overlayed with about 100 μl mineral oil.

The samples were subjected to 9 cycles of amplification in the Peltier device described in the previous example using the following program:

(1) 1 min., 35 to 60° C. ramp;

(2) 12 min., 60 to 70° C. ramp (extend);

(3) 1 min., 70–95° C. ramp (denature);

(4) 30 sec., 95° C. soak;

(5) 1 min., 95 to 35° C. ramp (anneal); and

(6) 30 sec., 35° C. soak.

After the last cycle, the samples were incubated an additional 10 minutes at 70° C. to complete the final (10th cycle) extension. The final volume of each was about 100 v1.

As before, 1/20th (10 u1) of each sample was analyzed on a agarose gel. In this gel, amplification products were present in all three samples: 110-bp for PCO3/PCO4, about 370-bp for RS_{45}/oligo(dT), and about 600-bp for PCO3/oligo(dT). These results were confirmed by Southern transfer and hybridization with the pBR328:betaA probe.

The production of the 600-bp product with Taq polymerase but not with the Klenow fragment is significant, and suggests that Taq polymerase is capable of producing longer DNA than the Klenow fragment.

EXAMPLE V

The Taq polymerase purified as described in Example VI of U.S. Pat. No. 4,889,818, supra, was diluted in a buffer described in that same copending U.S. application.

A reaction buffer was then prepared containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each dNTP, 1 μM each of the primers that define a 500 base pair target sequence on a control template from bacteriophage λ, and 2.0–2.5 units Taq polymerase/assay in a final volume of 100 μl. Template was added to the reaction buffer, the sample placed in a 0.5 ml polypropylene tube, and the sample topped with 100 μl of heavy white mineral oil to prevent evaporation. [One unit of enzyme activity is defined as the amount of enzyme that will incorporate 10 nmoles of dNTPs into acidinsoluble material in 30 minutes at 74° C. Enzyme activity was assayed using the conditions of 25 mM TAPS-Cl pH 9.3, 50 mM KCl, 2 mM $MgCl_{2,\ 1}$ mM β-mercaptoethanol, 200 μM each dATP, dGTP, and TTP, 100 μM dCTP (mix cold and [α-32P]), 250 μg activated salmon sperm DNA, in final volume of 50 μl.]

At least a 10^5-fold amplification was achieved when the following cycling conditions were employed, using 1 ng of control template (bacteriophage X DNA) where the target sequence represented approximately 1% of the starting mass of DNA.

First the template mixture was denatured for one minute, 30 seconds at 94° C. by placing the tube in a heat bath. Then the tube was placed in a heat bath at 37° C. for two minutes. Then the tube was placed in a heat bath at 72° C. for three minutes, and then in the heat bath at 94° C. for one minute. This cycle was repeated for a total of 25 cycles. At the end of the 25th cycle, the heat denaturation step at 94° C. was omitted and replaced by extending the 72° C. incubation step by an additional three minutes. Following termination of the assay, the samples were allowed to cool to room temperature and analyzed as described in previous examples.

The template may be optimally amplified with a different concentration of dNTPs and a different amount of Taq polymerase. Also, the size of the target sequence in the DNA sample will directly impact the minimum time required for proper extension (72° C. incubation step). An optimization of the temperature cycling profile should be performed for each individual template to be amplified, to obtain maximum efficiency.

EXAMPLE VI

Several 1 μg samples of human genomic DNA were subjected to 20–35 cycles of amplification as described in Example V, with equivalent units of either Klenow fragment or Taq polymerase, and analyzed by agarose gel electrophoresis and Southern blot. The primers used in these reactions, PCO3 and PCO4, direct the synthesis of a 110-bp segment of the human beta-globin gene. The Klenow polymerase amplifications exhibited the smear of DNA typically observed with this enzyme, the apparent cause of which is the nonspecific annealing and extension of primers to unrelated genomic sequences under what were essentially non-stringent hybridization conditions (1×Klenow salts at 37° C.). Nevertheless, by Southern blot a specific 110-bp beta-globin target fragment was detected in all lanes. A substantially different electrophoretic pattern was seen in the amplifications done with Taq polymerase where the single major band is the 110-bp target sequence. This remarkable specificity was undoubtedly due to the temperature at which the primers were extended.

Although, like Klenow fragment amplifications, the annealing step was performed at 37° C., the temperature of Taq-catalyzed reactions had to be raised to about 70° C. before the enzyme exhibited significant activity.

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During this transition from 37 to 70° C., poorly matched primer-template hybrids (which formed at 37° C.) disassociated so that by the time the reaction reached an enzyme-activating temperature, only highly complementary substrate was available for extension. This specificity also results in a greater yield of target sequence than similar amplifications done with Klenow fragment because the nonspecific extension products effectively compete for the polymerase thereby reducing the amount of 110-mer that can be made by the Klenow fragment.

EXAMPLE VII

Amplification was carried out of a sample containing 1 μg Molt 4 DNA, 50 mM KCl, 10 mM Tris pH 8.3, 10 mM $MgCl_2$, 0.01% gelatin, 1 μM of each of the following primers (to amplify a 150 bp region):

5'-CATGCCTCTTTGCACCATTC-3'(RS79) and 5'-TGGTAGCTGGATTGTAGCTG-3'(RS80)

1.5 mM of each dNTP, and 5.0 units of Taq polymerase per 100 μl reaction volume. Three additional samples were prepared containing 2.5, 1.3, or 0.6 units of Taq polymerase. The amplification was carried out in the temperature cycling machine described above using the following cycle, for 30 cycles:

from 70° to 98° C. for 1 minute
hold at 98° C. for 1 minute
from 98° C. to 35, 45 or 55° C. for 1 minute
hold at 35, 45 or 55° C. for 1 minute
from 35, 45 or 55° C. to 70° C. for 1 minute
hold at 70° C. for 30 seconds

At 35° C. annealing temperature, the 2.5 units/100 μl Taq enzyme dilution gave the best-signal-to noise ratio by agarose gel electrophoresis over all other Taq polymerase concentrations. At 45° C., the 5 units/100 μl Taq enzyme gave the best signal-to-noise ratio over the other concentrations. At 55° C., the 5 units/100 μl Taq enzyme gave the best signal-to-noise ratio over the other concentrations and over the 45° C. annealing and improved yield. The Taq polymerase has more specificity and better yield at 55° C.

In a separate experiment the Molt 4 DNA was 10-fold serially diluted into the cell line GM2064 DNA, containing no β- or δ-globin sequences, available from the Human Genetic Mutant Cell Depository, Camden, New Jersey, at various concentrations representing varying copies per cell, and amplification was carried out on these samples as described in this example at annealing temperatures of 35° C. and 55° C. At 35° C., the best that can be seen by agarose gel electrophoresis is 1 copy in 50 cells. At 55° C., the best that can be seen is 1/5,000 cells (a 100-fold improvement over the lower temperature), illustrating the importance of increased annealing temperature for Taq polymerase specificity under these conditions.

In a third experiment, DNA from a cell line 368H containing HIV-positive DNA, available from B. Poiesz, State University of New York, Syracuse, NY, was similarly diluted into the DNA from the SCI cell line (deposited with ATCC on Mar. 19, 1985; an EBV-transformed 8 cell line homozygous for the sickle cell allele and lacking any HIV sequences) at various concentrations representing varying copies per cell, and amplification was carried out as described in this Example at annealing temperatures of 35° C. and 55° C., using the primers SK38 and SK39, which amplify a 115 bp region of the HIV sequence:

5'-ATAATCCACCTATCCCAGTAGGAGAAAT-3'(SK38) and 5'-TTTGGTCCTTGTCTTATGTCCAGAATGC-3'(SK39)

The results by agarose gel electrophoresis showed that only the undiluted 368H sample could be detected with the annealing temperature at 35° C., whereas at least a 10–2 dilution can be detected with the annealing temperature at 55° C., giving a 100-fold improvement in detection.

In summary, the present invention is seen to provide a process for amplifying one or more specific nucleic acid sequences using a temperature-cycled chain reaction and a thermostable enzyme, in which reaction primer extension products are produced that can subsequently act as templates for further primer extension reactions. The process is especially useful in detecting nucleic acid sequences that are initially present in only very small amounts and in detecting nucleotide variations using sequence-specific oligonucleotides. Also, the amplification process herein can be used for molecular cloning.

The process herein results in increased yields of amplified product, greater specificity, and fewer steps necessary to carry out the amplification procedure, over what has been previously disclosed.

Other modifications of the above-described embodiments of the invention that are obvious to those of skill in the area of molecular biology and related disciplines are intended to be within the scope of the following claims.

What is claimed is:

1. A process for amplifying at least one specific DNA sequence contained in a DNA or a mixture of nucleic acids, wherein if the DNA is double-stranded, it consists of two separated complementary strands of equal or unequal length, which process comprises:

(a) contacting the DNA with four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being amplified, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand;

(b) contacting each strand, at the same time as or after step (a), with thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme, and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand, but not so high as to separate

each extension product from its complementary strand;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(e) cooling the mixture from step (d) to an effective temperature to promote hybridization of each primer to each of the single-stranded molecules produced in step (d); and

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand produced in step (d), but not so high as to separate each extension product from its complementary strand, wherein steps (e) and (f) are conducted simultaneously or sequentially.

2. The process of claim **1**, wherein one specific DNA acid sequence is amplified and two primers are employed.

3. The process of claim **1**, wherein after steps (d)-(f) are repeated at least once, a second set of two primers is added, wherein the primers added are sufficiently complementary to different strands at internal sequences of the amplified sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer.

4. The process of claim **1**, wherein steps (d), (e) and (f) are repeated at least five times.

5. The process of claim **4**, wherein aid thermostable enzyme is a polymerase from *Thermus aquaticus.*

6. The process of claim **3** wherein in step (a) the triphosphates and primer(s) are contained in a buffer comprising 1.5–2 mM of a magnesium salt, 150–200 μM each of the triphosphates, and 1 μM 58° C., and step (d) is carried out at about 90–100° C.

7. The process of claim **1**, wherein said primers are oligodeoxyribonucleotides.

8. The process of claim **1**, wherein said DNA is cDNA.

9. The process of claim **1**, wherein the heating and cooling steps (d)-(f) are automated by a machine which controls temperature levels, transitions from one temperature to another, and the timing of temperature levels.

10. The process of claim **1**, wherein each primer is present in a molar ratio of at least 1000:1 primer:complementary strand.

11. The process of claim **1**, wherein at least one primer contains at least one nucleotide which is not complementary to the specific sequence to be amplified.

12. The process of claim **11**, wherein said primer that contains at least one nucleotide which is not complementary to the specific sequence to be amplified encodes a promoter.

13. The process of claim **1**, wherein steps (e) and (f) are carried out sequentially, steps (c) and (f) take place at 40–80° C., step (d) takes place at 90–105° C., and step (e) takes place at 35–65° C.

14. The process of claim **1** wherein steps (e) and (f) are carried out sequentially, steps (c) and (f) take place at 50–75° C., step (d) takes place at 90–100° C., and step (e) takes place at 37° C.–60° C.

15. The process of claim **1** wherein steps (e) and (f) are carried out simultaneously at about 45–70° C.

16. The process of claim **1**, wherein said specific DNA sequence is contained in a mixture of nucleic acids.

17. The process of claim **16**, wherein said specific DNA sequence is contained in a larger sequence.

18. The process of claim **1**, wherein said specific DNA sequence is contained in a larger sequence.

19. A process for detecting the presence of a specific DNA sequence in a sample, said process comprising:

(a) amplifying said sequence by the process of claim **1**; and

(b) determining if amplification has occurred.

20. A process for amplifying at least one specific DNA sequenc contained in a DNA or a mixture of nucleic acids, wherein the DNA consists of two complementary strands of equal or unequal length, which process comprises:

(a) heating the DNA in the presence of four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequencing being amplified, for an effective time and at an effective temperature to denature each nucleic acid, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) cooling the denatured DNA to temperature which promotes hybridization of each primer to its complementary strand;

(c) contacting the denatured DNA, at the same time as or after step (a) or (b), with a thermostable enzyme which enables combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(d) maintaining the mixture from step (c) at an effective temperature for an effective time to promote the activity of the thermostable enzyme, and to synthesize, for each different sequence being amplified, an extension product of each primer which si complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(e) heating the mixture from step (d) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(f) cooling the mixture from step (e) for an effective time and to an effective temperature to promote hybridization of the primer to its complementary single-stranded molecule produced in step (e); and

(g) maintaining the mixture from step (f) at an effective temperature for an effective time to promote the activity of the enzyme, and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand produced in step (f), but not so high as to separate each extension product form its complementary strand, wherein steps (f) and (g) are carried out simultaneously or sequentially.

21. The process of claim 20, wherein said specific DNA sequence is contained in a mixture of nucleic acids.

22. The process of claim 21, wherein said specific DNA sequence is contained in a

23. The process of claim 20, wherein said specific DNA sequence is contained in a larger sequence.

24. A process for detecting the presence of a specific DNA sequence in a sample, said process comprising:

(a) amplifying said sequence by the process of claim 20, and

(b) determining if amplification has occurred.

25. A process for detecting the presence or absence of at least one specific DNA sequence in a sample containing a DNA or mixture of nucleic acids, or distinguishing between two different DNA sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, and wherein if the nucleic acid(s) are double-stranded, they each consist of two separated complementary strands of equal or unequal length, which process comprises:

(a) contacting the sample with four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being detected, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, at a temperature which promotes hybridization of each primer to its complementary strand;

(b) contacting the sample, at the same time as or after step (a), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme, and to synthesize, for each different sequence being detected, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the thermostable enzyme;

(e) cooling the mixture from step (d) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced in step (d);

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being detected, but not so high as to separate each extension product from its complementary strand, resulting in amplification in quantity of the specific nucleic acid sequence or sequences if present, wherein steps (e) and (f) are carried out simultaneously or sequentially;

(g) adding to the product of step (f) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to said sequence or to a mutation thereof; and

(h) determining whether said hybridization has occurred.

26. A process for detecting the presence or absence of at least one specific DNA sequence in a sample containing DNA or mixture of nucleic acids, or distinguishing between two different DNA sequences in said sample, where the sample is suspected of containing said sequence or sequences and the DNA is double-stranded, which process comprises:

(a) heating the sample in the presence of four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being detected, for an effective time and at an effective temperature to denature the DNA in the sample, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer,

(b) cooling the denatured DNA to a temperature promotes hybridization of each primer to its complementary strand;

(c) contacting the natured DNA, at the same time as or after step (a) or (b), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(d) maintaining the mixture from step (c) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being detected, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(e) heating the mixture from step (d) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(f) cooling the mixture from step (e) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced from step (e);

(g) maintaining the mixture from step (f) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being detected, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand, resulting in amplification in quantity of the specific nucleic acid sequence or sequences if present, wherein steps (f) and (g) are carried out simultaneously or sequentially;

(h) adding to the product of step (g) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to said sequence or to a mutuation thereof; and

(i) determining whether said hybridizing has occurred. determining whether said hybridization has occurred.

27. The process of claim 26, wherein one specific DNA sequence is being detected and two primers are employed, and before step (a) the DNA is extracted from the sample.

28. The process of claim 26, wherein steps (d), (e) and (f) are repeated at least 20 times.

29. The process of claim 26, wherein said thermostable enzyme is a polymerase from Thermus aquaticus.

30. The process of claim 26, wherein said primers are oligodeoxyribonucleotides.

31. The process of claim 26, wherein said DNA is cDNA.

32. The process of claim 26, wherein the heating and cooling steps (e)-(g) are automated by a machine which controls temperature levels, transitions from one temperature to another, and the timing of temperature levels.

33. The process of claim 26, wherein the specific DNA sequence to be detected can cause a genetic, infectious or cancerous disease.

34. The process of claim 33, wherein the genetic disease is sickle cell anemia or hemoglobin C disease.

35. The process of claim 34, wherein after step (g) and before step (h) the sample is cut with a restriction enzyme and electrophoresed, and step (i) is accomplished by Southern blot analysis.

36. A process for cloning into a cloning vector one or more specific DNA sequences contained in a DNA or a mixture of nucleic acids, which DNA when double-stranded consists of two separated complementary strands, and which DNA is amplified in quantity before cloning, which process comprises:

(a) contacting each DNA with four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being amplified, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each sequence being amplified or each primer contains a restriction site, at a temperature which promotes hybridization of each primer to its complementary strand;

(b) contacting each strand, at the same time as or after step (a) or (b), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized, to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(e) cooling the mixture from step (d) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced in step (d);

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand produced in step (d), but not so high as to separate each extension product from its complementary strand, steps (d), (e) and (f) being repeated a sufficient number of times to result in detectable amplification of the DNA containing the sequence(s), wherein steps (e) and (f) are carried simultaneously or sequentially;

(g) adding to the product of step (f) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(h) ligating the cleaved product(s) of step (g) containing the specific sequence(s) to be cloned into one or more cloning vectors.

37. A process for cloning into a vector at least one specific DNA sequence contained in a DNA or a mixture of nucleic acids, wherein the DNA consists of two complementary strands of equal or unequal length, and wherein the DNA is amplified in quantity before cloning, which process comprises:

(a) heating the DNA in the presence of four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being amplified, for an effective time and at an effective temperature to denature the DNA, wherein each primer is selected to be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each sequence being amplified or each primer contains a restriction site;

cooling the denatured DNA to a temperature effective to promote hybridization between each primer and its complementary strand;

(c) contacting the denatured DNA, at the same time as or after step (a) or (b), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(d) maintaining the mixture from step (c) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(e) heating the mixture from step (d) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized, to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(f) cooling the mixture from step (e) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced in step (e);

(g) maintaining the mixture from step (f) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complemen-

tary to each strand produced in step (e), but not so high as to separate each extension product from its complementary strand, steps (e), (f), and (g) being repeated a sufficient number of times to result in detectable amplification of the DNA containing the sequences(s), wherein steps (f) and (g) are carried out simultaneously or sequentially;

(h) adding to the product of step (g) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(i) ligating the cleaved product(s) of step (h) containing the specific sequence(s) to be cloned into one or more cloning vectors containing a selectable marker.

38. The process of clam **37**, wherein steps (e)-(g) are repeated at least five times.

39. The process of claim **37**, further comprising the step of passing the restriction digest of step (h) through a desalting column or membrane before step (i).

40. The process of claim **37**, further comprising, after step (i), sequencing the cleaved product ligated into the vector.

41. The process of claim **37**, wherein said specific DNA sequence encodes a protein and wherein said method further comprises, after step (i), expressing the protein encoded by the specific nucleic acid sequence.

42. The process of claim **37**, wherein one specific sequence is being amplified, the restriction sites are different on each primer, and the product of step (h) is ligated into one cloning vector with a specific orientation.

43. The process of claim **42**, wherein the specific DNA sequence amplified is or is contained within the β-globin gene or the N-RAS oncogene.

44. The process of claim **37**, wherein the thermostable enzyme is a polymerase from Thermus aquaticus.

45. The process of claim **37**, wherein the heating and cooling steps (e)-(g) are automated by a machine which controls temperature levels, transitions from one temperature to another, and the timing of the temperature levels.

46. The process of claim **36**, wherein each primer contains a restriction site which is the same as or different from the restriction site(s) on the other primer(s).

47. The process of clam **46**, wherein the restriction sites are on the 5′ end of the primers.

48. A process for cloning into a cloning vector one or more specific DNA sequences contained in a DNA or mixture of nucleic acids, which DNA when double-stranded consists of two separated complementary strands of equal to unequal length and which DNA is amplified in quantity before cloning which process comprises:

(a) contacting each DNA with four different nucleoside triphosphates and two oligonucleotide primers, for each different specific sequence being amplified, wherein each primer is selected to sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separatd from its complement, can serev as a template for synthesis of teh extension product of teh other primer, at a temperature which promotes hybridization of each primer to its complementary strand;

(b) contacting each strand, at the same time as or after step (a) or (b), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(c) maintaining the mixture from step (b) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(d) heating the mixture from step (c) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(e) cooling the mixture from step (d) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced in step (d);

(f) maintaining the mixture from step (e) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand produced in step (d), but not so high as to separate each extension product from its complementary strand, steps (d), (e) and (f) being repeated a sufficient number of times to result in effective amplification of the DNA containing the sequence(s) for blunt-end ligation into one or more cloning vectors, wherein steps (e) and (f) are conducted simultaneously or sequentially; and

(g) ligating the amplified specific sequence(s) to be cloned obtained from step (f) into one or more of said cloning vectors in the presence of a ligase, said amplified sequence(s) and vector(s) being present in sufficient amounts to effect the ligation.

49. A process for cloning into a cloning vector at least one specific DNA sequence contained in a DNA or mixture of nucleic acids, which DNA consists of two complementary strands of equal to unequal length and which DNA is amplified in quantity before cloning, which process comprises:

(a) heating the DNA in the presence of four different nucleoside triphophates and two oligonucleotide primers, for each different specific sequencing being amplified, for an effective time and at an effective temperature to denature the DNA, wherein each primer is selected be sufficiently complementary to different strands of each specific sequence to hybridize therewith, such that the extension product synthesized from one primer, when separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) cooling the denatured DNA to a temperature effective to promote hybridization between each primer and its complementary strand;

(c) contacting the denatured DNA, at the same time as or after step (a) or (b), with a thermostable enzyme which catalyzes combination of the nucleoside triphosphates to form primer extension products complementary to each strand of DNA;

(d) maintaining the mixture from step (c) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an exten-

sion product of each primer which is complementary to each strand, but not so high as to separate each extension product from its complementary strand;

(e) heating the mixture from step (d) for an effective time and at an effective temperature to separate the primer extension products from the strands on which they were synthesized, to produce single-stranded molecules, but not so high as to denature irreversibly the enzyme;

(f) cooling the mixture from step (e) for an effective time and to an effective temperature to promote hybridization of each primer to its complementary single-stranded molecule produced in step (e);

(g) maintaining the mixture from step (f) at an effective temperature for an effective time to promote the activity of the enzyme and to synthesize, for each different sequence being amplified, an extension product of each primer which is complementary to each strand produced in step (e), but not so high as to separate each extension product from its complementary strand, steps (e), (f) and (g) being repeated a sufficient number of times to result in effective amplification of the DNA containing each sequence for bluntend ligation into one or more cloning vectors, wherein steps (f) and (g) are conducted simultaneously or sequentially; and

(h) ligating the amplified specific sequence(s) to be cloned obtained from step (g) into one or more of said cloning vectors in the presence of a ligase, said amplified sequence(s) and vector(s) being present in sufficient to effect the ligation.

50. The process of claim **49**, wherein one DNA sequence is cloned into one vector and two primers are employed.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,965,188

Page 1 of 5

DATED : October 23, 1990

INVENTOR(S) : Kary B. Mullis, Henry A. Erlich, David H. Gelfand, Glenn Horn, and Randall K. Saiki

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 4, line 28, change "primer.," to --primer;--.

Column 4, line 42, change "sequence(sl," to --sequence(s),--.

Column 6, line 25, change "$MgCl_{12}$," to --$MgCl_2$,--.

Column 6, line 27, change "pH 88.4," to --pH 8-8.4,--.

Column 7, line 9, change "8globin" to --β-globin--.

Column 10, line 42, after "any" delete ".".

Column 10, line 47, change "10^6 1" to --10^6:1--.

Column 11, line 6, after "preferably" delete "room".

At column 11, line 14, and throughout the specification, change "oligonucleoside" to --oligonucleotide--.

Column 11, line 52, after "complementary" delete ".".

Column 12, line 8, change "80° C. 90° C.)." to --80°C.-90°C.).--.

Column 12, line 68, after "amplify" delete ",".

Column 13, line 7, after "amplify" delete ",".

Column 13, line 35, change "CHC_{13}." to --$CHCl_3$,--.

Column 14, line 48, change "[S^{31}]." to --[S-].--.

Column 17, line 39, change "products" to --product--.

Column 17, line 63, change "rheterozygous" to --heterozygous--.

Column 19, line 55, change "way.," to --way,--.

Column 20, line 28, change "u-Thalassemia" to --α-Thalassemia--.

Column 20, line 29, change "βtha-" to --β-tha---.

At column 21, line 38, and throughout the specification, change "nucleoside variation" to --nucleotide variation--.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,965,188 Page 2 of 5

DATED : October 23, 1990

INVENTOR(S) : Kary B. Mullis, Henry A. Erlich, David H. Gelfand, Glenn Horn, and Randall K. Saiki

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

At column 22, line 17, and throughout the specification, change "nucleoside sequence" to --nucleotide sequence--.

At column 22, line 24, and throughout the specification, change "class 11" to --class II--.

Column 22, line 39, change "HLA-DR-βthree" to --HLA-DR-β, three--.

Column 22, line 54, change "C_1-6)," to --C1-6),--.

Column 23, line 11, change "nucleoside composition" to --nucleotide composition--.

Column 23, line 13, change "15-25 nucleosides" to --15-25 nucleotides--.

Column 23, line 14, change "nucleosides" to --nucleotides--.

Column 24, line 31, change "α0" to --α--.

Column 24, line 31, change "9enes" to --genes--.

Column 24, line 38, change "melliters," to --mellitus,--.

Column 25, line 4, change "3,879" to --63,879--.

Column 25, line 15, change "copending" to --now abandoned--.

Column 28, line 7, directly following "activity" delete ".".

Column 29, line 15, change "$PC_{04,\ 1.5}$ mM" to --PC04, 1.5 mM--.

Column 29, line 39, change "RS_{24}," to --RS24,--.

Column 29, line 66, change "a ml" to --a 1 ml--.

Column 30, line 2, change "vCi/pmole" to --μCi/pmole--.

Column 30, line 24, change "albumin,," to --albumin,--.

Column 30, line 27, change "RS_{24}" to --RS24--.

Column 30, line 48, change "pBR.βA" to --pBR:β^A--.

Column 30, line 48, change "βA" to --β^A--.

UNITED STATES PATENT AND TRADEMARK OFFICE

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INVENTOR(S) : Kary B. Mullis, Henry A. Erlich, David H. Gelfand, Glenn Horn, and Randall K. Saiki

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 30, line 59, change "$MgCl_{12,\ 1}$" to --$MgCl_2$, 1--.

Column 30, line 60, change "PCO_3 1 nM" to --PCO3, 1 μM--.

Column 30, line 60, change "$PCO_{4,\ 200}$" to --PCO4, 200--.

Column 31, line 6, change "minutes.," to --minutes;--.

Column 31, line 8, change "anneal.," to --anneal;--.

Column 31, line 34, change "RS_{18}," to --RS18,--.

Column 31, line 40, change "μ-globin" to --β-globin--.

Column 31, line 41, change "(μA)." to --(β^A).--.

Column 31, line 63, change "RS_{18}" to --RS18--.

Column 32, line 8, after "20 mM" and before "and", insert --EDTA), as disclosed by Reed--.

Column 32, line 51, delete "Z' and if the lower".

Column 32, line 52, after "each," insert --and if the lower--.

Column 32, line 61, change "μ-globin" to --β-globin--.

Column 33, line 19, change "4 nl" to --4 μl--.

Column 33, line 35, change "$_{20}$ units" to --20 units--.

Column 33, line 41-42, change "μ-globin" to --β-globin--.

Column 33, line 54, change "XDNA" to --λ DNA--.

Column 33, line 55, change "μlat" to --μl at--.

Column 33, line 59, change "Molt digested λDNA" to --Molt 4 DNA--.

Column 34, line 1, change "μ-globin" to --β-globin--.

Column 34, line 39, change "oligo(dT)1218" to --oligo$(dT)_{12\text{-}18}$--.

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DATED : October 23, 1990

INVENTOR(S) : Kary B. Mullis, Henry A. Erlich, David H. Gelfand, Glenn Horn, and Randall K. Saiki

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 34, line 51, and throughout the specification, change "RS_{45}" to --RS45--.

Column 34, line 51, change "PCO_3" to --PCO3,--.

Column 34, line 56, change " 5"- " to -- 5'- -- and " -3" " to -- -3'--.

Column 35, line 18, change "8globin" to --β-globin--.

Column 35, line 22, change "μ-globin" to --β-globin--.

Column 35, line 49, change "100 vl" to --100 μl--.

Column 36, line 10, change "acidinosoluble" to --acid insoluble--.

Column 36, line 19, change "X DNA" to --λ DNA--.

Column 37, line 67, change "8 cell" to --β cell--.

Column 38, line 16, change "10-2" to --10^{-2}--.

Claim 5, line 1, change "aid" to --said--.

Claim 6, line 1, change "claim 3" to --claim 5--.

Claim 6, line 4, after "μM" and before "58°C.,", insert --of each primer, steps (a), (e) and (f) are carried out at about 45° - --.

Claim 20, line 2, change "sequenc" to --sequence--.

Claim 20, step (b), line 1, after "to" and before "temperature" insert --a--.

Claim 20, step (g), line 7, change "form" to --from--.

Claim 22, line 2, after "a", insert --larger sequence.--.

Claim 25, step (f), line 4, after "detected," and before "but", insert --an extension product of each primer which is complementary to each strand, --.

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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INVENTOR(S) : Kary B. Mullis, Henry A. Erlich, David H. Gelfand, Glenn Horn, and Randall K. Saiki

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Claim 26, line 3, after "ing" and before "DNA", insert --a--.

Claim 26, line 5, change "where" to --wherein--.

Claim 26, step (c), line 1, change "natured" to --denatured--.

Claim 26, step (i), lines 1 and 2, delete "determining whether said hybridizing has occurred.".

Claim 36, step (f), last line, after "ried" and before "simultaneously", insert --out--.

Claim 37, step (a), line 14, before the unindented word "cooling", insert --(b)--.

Claim 46, line 1, change "claim 36," to --claim 37,--.

Claim 48, line 5, change "to" to --or--.

Claim 48, line 6, insert --,-- after "cloning".

Claim 48, step (a), line 8, change "separatd" to --separated--, and change "serev" to --serve--.

Claim 48, step (a), line 9, change "teh" to --the--.

Claim 48, step (a), line 10, change "teh" to --the--.

Claim 49, line 4, change "to" to --or--.

Claim 49, step (h), line 5, after "sufficient" and before "to", insert --amounts--.

Signed and Sealed this

Ninth Day of June, 1992

Attest:

DOUGLAS B. COMER

Attesting Officer *Acting Commissioner of Patents and Trademarks*

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

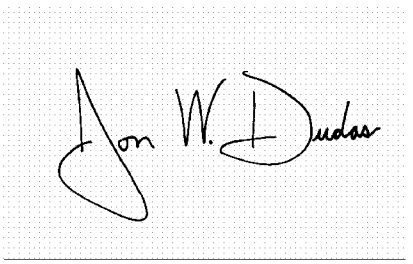
PATENT NO. : 4,965,188
DATED : October 23, 1990
INVENTOR(S) : Kary B. Mullis et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
After [*] Notice, please replace "The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed." with -- This patent is subject to a terminal disclaimer. --

Signed and Sealed this

Tenth Day of August, 2004



JON W. DUDAS
Acting Director of the United States Patent and Trademark Office

United States Patent [19]

Blanco et al.

[11] **Patent Number: 5,001,050**

[45] **Date of Patent: Mar. 19, 1991**

[54] **PHϕ29 DNA POLYMERASE**

[75] Inventors: **Luis Blanco; Antonio Bernad; Margarita Salas,** all of Madrid, Spain

[73] Assignee: **Consejo Superior Investigaciones Cientificas,** Madrid, Spain

[21] Appl. No.: **328,462**

[22] Filed: **Mar. 24, 1989**

[51] **Int. Cl.**[5] **C12Q 1/70;** C12N 9/12; G01N 33/566; C12P 19/34

[52] **U.S. Cl.** **435/5;** 435/6; 435/91; 435/19.9; 435/183; 435/172.3; 436/501; 436/93

[58] **Field of Search** 435/6, 91, 194, 810, 435/5; 436/501; 935/77, 78

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(List continued on next page.)

Primary Examiner—Robert A. Wax
Assistant Examiner—Stephanie W. Zitomer
Attorney, Agent, or Firm—Fish & Richardson

[57] **ABSTRACT**

An improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is provision of a DNA-polymerase which is a ϕ29-type DNA polymerase.

20 Claims, 2 Drawing Sheets

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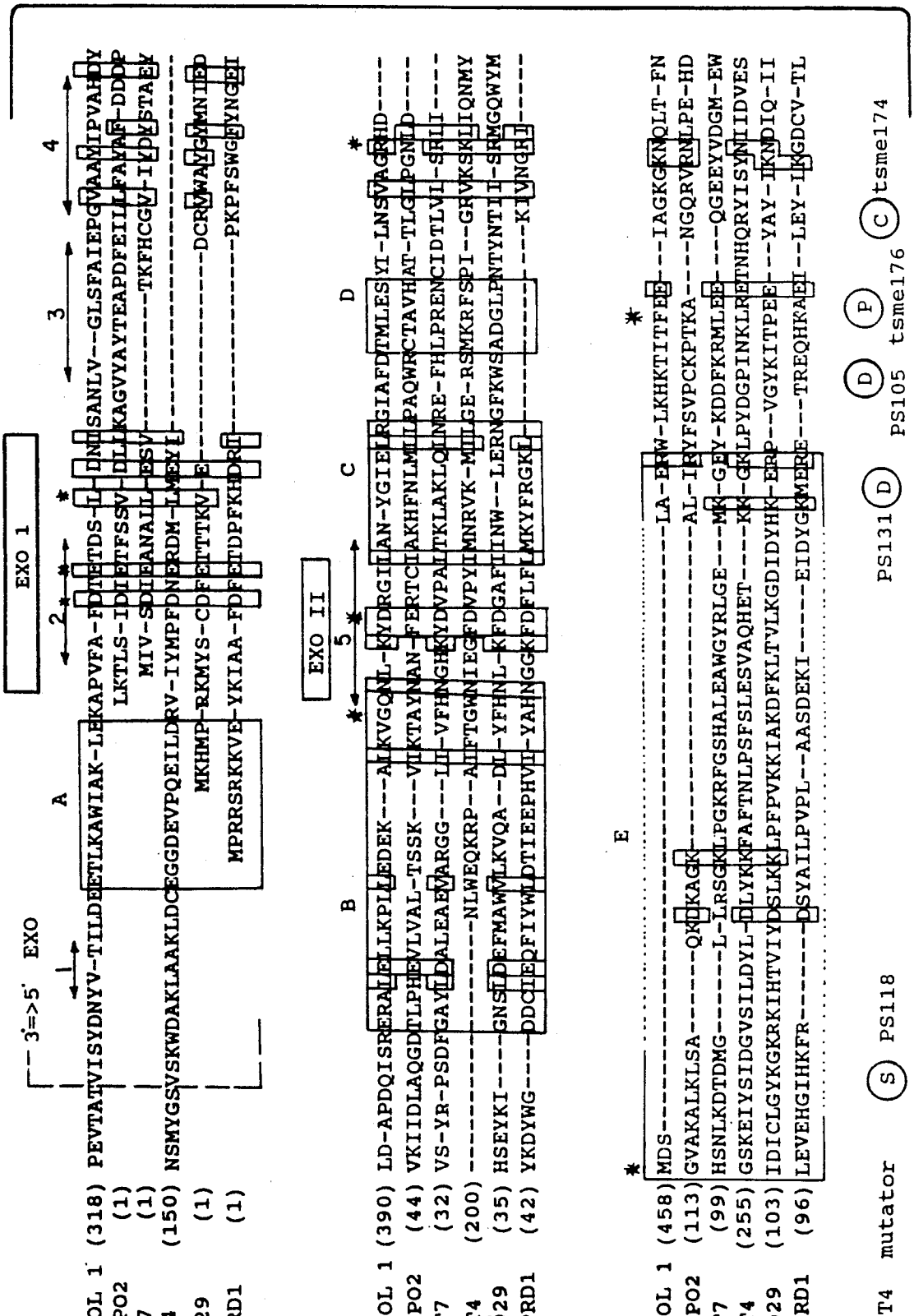
EXO 1
EXO II
3'=>5' EXO
A
B
C
D
E
1
2
3
4
5
POL 1 (318) PEVTATVISYDNYV-TILDEETLKAWIAK-LEKAPVFA-FDTETDS-LDNISANLV--GLSFAIEPGVAAAMIPVAHDY
SPO2 (1) LKTLS-IDIETFSSV-DLLKAGVYAYTEAPDFEILLFAFAF-DDGP
T7 (1) MIV-SDIEANAIL-ESV----------TKFHCGV-IVDVSTAEV
T4 (150) NSMYGSVSKWDAKLAAKLDCEGGDEVPQEILDRV-IYMPFDNERDM-LMEYL----------
ϕ29 (1) MKHMP-RKMYS-CDFETTTKV-E----------DCRMWAVGIMNIED
PRD1 (1) MPRRSRKKVE-YKIAA-FDFETDPFKHDRL----------PKPFSWGEYNGEI
POL 1 (390) LD-APDQISRERALELLKPLEEDEK---AIKVGQNL-KVDRGILAN-YGIELRGIAFDTMLESYI-LNSVAGRHD----
SPO2 (44) VKIIDLAQGDTLPHEVLVAL-TSSK---VIKTAYNAN-FERTCIAKHFNLMILPAQWRCTAVHAT-TLGLPGNLD----
T7 (32) VS-YR-PSDFGAVLDALEAEVARGG---LI-VFHNGHKVDVPALTKLAKLQLNRE-FHLPRENCIDTLVI-SRLI----
T4 (200) ----------NLWEQKRP--AIFTGWNIEGFDVPYIMNRVK-MILGE-RSMKRFSPI--GRVKSKLIQNMY
ϕ29 (35) HSEYKI----GNSLDEFMAWVLKVQA--DL-YFHNL-KFDGAFIINW--LERNGFKWSADGLPNTYNTII-SRMGQWYM
PRD1 (42) YKDYWG----DDCIEDFIYWLDTIEEPHVI-YAHNGGKFDFLFLMKYFRGKL----------KIVNGRI----
POL 1 (458) MDS----------LA-ERW-LKHKTITFEE--IAGKGKNQLT-FN
SPO2 (113) GVAKALKLSA----------QKDKAGK----------AL-IRYFSVPCKPTKA----NGQRVRNLPE-HD
T7 (99) HSNLKDTDMG----------L-LRSGKLPGKRFGSHALEAWGYRLGE---MK-GEY-KDDFKRMLEE---QGEEYVDGM-EW
T4 (255) GSKEIYSIDGVSILDYL-DLYKKFAFTNLPSFSLESVAQHET----KK-GKLPYDGPINKLRETNHQRYISYNIIDVES
ϕ29 (103) IDICLGYKGKRKIHTVIYDSLKKLPFPVKKIAKDFKLTVLKGDIDYHK-ERP--VGYKITPEE--YAY-IKNDIQ-II
PRD1 (96) LEVEHGIHKFR----------DSYAILPVPL--AASDEKI----EIDYGKMERE---TREQHKAEI--LEY-LKGDCV-TL
T4 mutator S PS118 PS131 D PS105 tsme176 D P C tsme174

FIG. 1

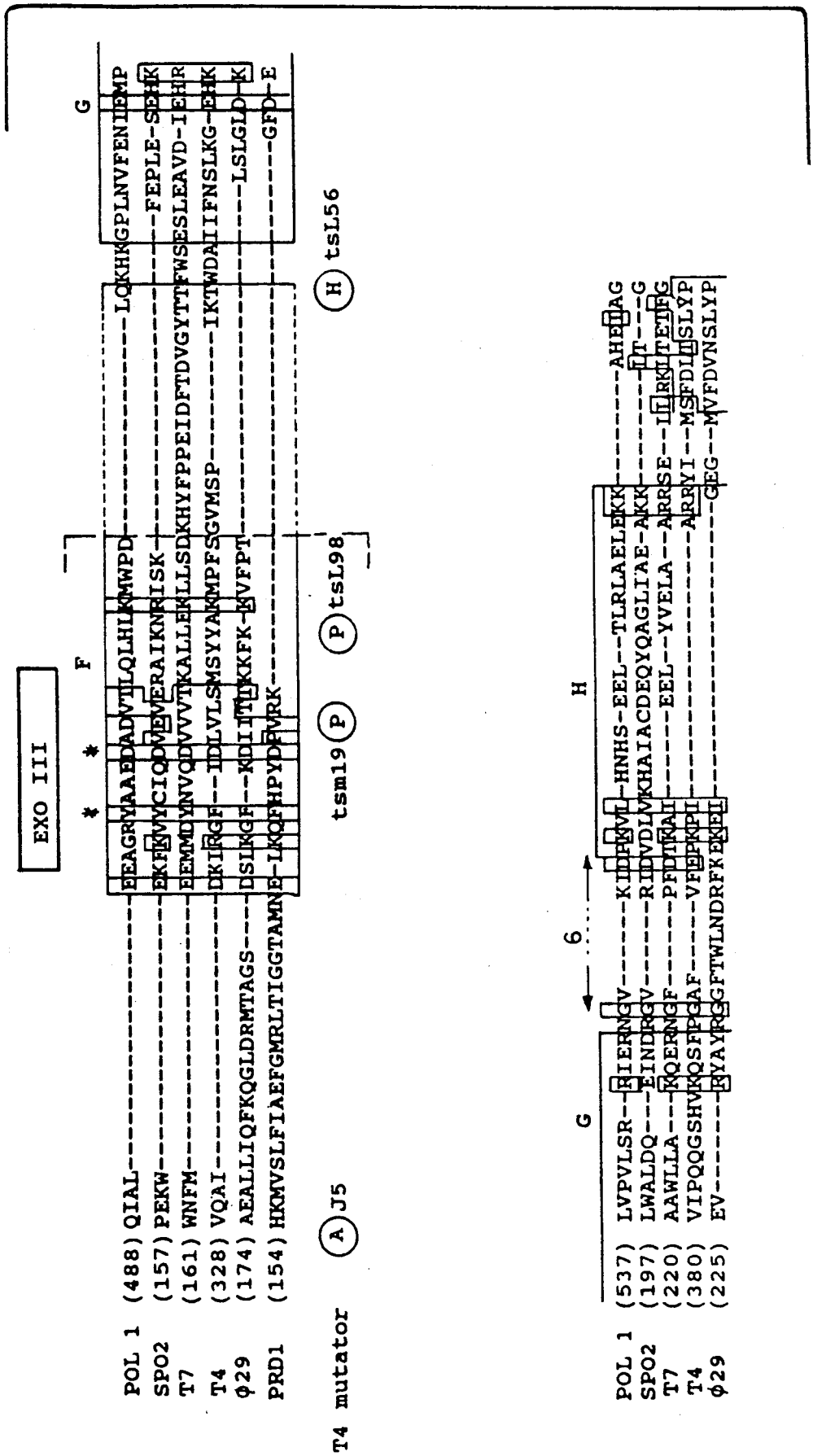
EXO III
A J5
T4 mutator
tsm19 P
P tsL98
H tsL56
POL 1 (488) QIAL
SPO2 (157) PEKW
T7 (161) WNFM
T4 (328) VQAI
φ29 (174) AEALLIQFKQGLDRMTAGS
PRD1 (154) HKMVSLFIAEFGMRLTIGGTAMN
POL 1 (537) LVPVLSR
SPO2 (197) LWALDQ
T7 (220) AAWLLA
T4 (380) VIPQQGSHVKQSFPGAF
φ29 (225) EV

FIG. 1 CONT

PHϕ29 DNA POLYMERASE

BACKGROUND OF THE INVENTION

This invention relates to DNA polymerases suitable for DNA sequencing.

DNA sequencing involves the generation of four populations of single-stranded DNA fragments, having one defined terminus and one variable terminus. The variable terminus always terminates at a specific given nucleotide base (either guanine (G), adenine (A), thymine (T), or cytosine (C)). The four different sets of fragments are each separated on the basis of their length, on a high resolution polyacrylamide gel; each band on the gel corresponds colinearly to a specific nucleotide in the DNA sequence, thus identifying the positions in the sequence of the given nucleotide base.

Generally there are two methods of DNA sequencing. One method (Maxam and Gilber sequencing) involves the chemical degradation of isolated DNA fragments, each labeled with a single radiolabel at its defined terminus, each reaction yielding a limited cleavage specifically at one or more of the four bases (G, A, T or C). The other method (dideoxy sequencing) involves the enzymatic synthesis of a DNA strand. Four separate syntheses are run, each reaction being caused to terminate at a specific base (G, A, T or C) via incorporation of the appropriate chain terminating dideoxynucleotide. The latter method is preferred since the DNA fragments are uniformly labelled (instead of end labelled) and thus the larger DNA fragments contain increasingly more radioactivity. Further, ^{35}S-labelled nucleotides can be used in place of ^{32}P-labelled nucleotides, resulting in sharper definition; and the reaction products are simple to interpret since each lane corresponds only to either G, A, T or C. The enzymes used for most dideoxy sequencing are the *Escherichia coli* DNA-polymerase I large fragment ("Klenow"), AMV reverse transcriptase, and T7 DNA polymerase (Tabor et al., U.S. Pat. No. 4,795,699). The T7 DNA polymerase used for sequencing is said to be advantageous over other DNA polymerases because it is processive, has no associated exonuclease activity, does not discriminate against nucleotide analog incorporation, and can utilize small oligonucleotides as primers. These properties are said to make the polymerase ideal for DNA sequencing. Id.

SUMMARY OF THE INVENTION

In a first aspect, the invention features an improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is provision of a DNA polymerase which is a ϕ29-type DNA polymerase.

By ϕ29-type DNA polymerase is meant any DNA polymerase isolated from the related phages which contain a terminal protein used in the initiation of replication of DNA. These phages are generally described by Salas, 1 The Bacteriophages 169, 1988. These phages are closely related in the structure of their DNA polymerases, some differing by as few as 6 amino acid changes with 5 of those amino acids being replaced by similar amino acids. These phages have a short inverted terminal repeat sequence of length between about 6 and 300 nucleotides. These polymerases have a highly active 3′–5′ exonuclease activity, but no 5′–3′ exonuclease activity. Surprisingly, although they are related to the T4 family of DNA polymerases, they are able to adequately recognize chain terminating agents such as dideoxynucleosides and therefore are useful for DNA sequencing. This ability is even more surprising since the exonuclease is known to recognize both deoxy and dideoxy ADP. Blanco et al. 13 Nuc. Acid. Res. 1239, 1246, 1985.

In preferred embodiments, the ϕ29-type DNA polymerase is either that phage polymerase in cells infected with a ϕ29-type phage; the ϕ29-type DNA polymerase is chosen from the DNA polymerases of phages: ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; or the DNA polymerase is a ϕ29-type polymerase modified to have less than ten percent of the exonuclease activity of the naturally-occurring polymerase, most preferably the polymerase has less than one percent, and even more preferably has substantially no exonuclease activity; and the terminating agent is a dideoxynucleotide.

In a related aspect, the invention features a kit for DNA sequencing including a supply of ϕ29-type DNA polymerase, together with a supply of a chain terminating agent. By kit is meant a container designed to keep these two components separated from each other, preferably in condition for use in a DNA sequencing reaction.

In another related aspect, the invention features a DNA fragment encoding a modified ϕ29-type DNA polymerase, wherein the polymerase has sufficient DNA polymerase activity for use in DNA sequencing, and an exonuclease activity which is less than 10% the activity of the corresponding naturally occurring ϕ29-type DNA polymerase.

By corresponding is meant that the modified polymerase is derived from a naturally occurring polymerase, generally by in vitro mutagenesis of the DNA sequence encoding the latter polymerase, and the latter is the corresponding polymerase.

In preferred embodiments, the DNA fragment is modified to substantially eliminate the naturally-occurring exonuclease activity; and the DNA fragment includes a DNA sequence encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14, or 16 of the polymerase is replaced by an alanine moiety.

The invention also features a ϕ29-type DNA polymerase produced from the above described DNA fragments.

In another aspect, the invention features an improved method for amplification of a DNA sequence. The method includes annealing a first and second primer to opposite strands of a double-stranded DNA sequence, and incubating the annealed mixture with a DNA polymerase. The improvement includes employing as the DNA polymerase a ϕ29-type DNA polymerase.

In preferred embodiments, the first and second primers have their 3′ ends directed towards each other after

annealing; the method further includes, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the denatured DNA and incubating the last annealed mixture with the polymerase; the cycle of denaturing, annealing, and incubating is repeated from 10–40 times; the ϕ29-type DNA polymerase is selected from the DNA polymerases of phages: ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; the DNA polymerase exhibits less than 10% of the naturally-occurring exonuclease activity exhibited by the corresponding naturally-occurring polymerase, most preferably the polymerase has no detectable exonuclease activity.

In a further aspect, the invention features a method for production of DNA molecules of greater than 10 kilobases in length. The method includes providing a template DNA molecule; annealing a primer with the template molecule; and incubating the annealed primer and template molecules in the presence of a ϕ29-type DNA polymerase, and a mixture of four different deoxynucleoside triphosphates.

The invention also features a method for amplification of a heterologous DNA molecule including covalently bonding a ϕ29-type terminal DNA sequence at one end of the DNA molecule to form a product; and incubating the product in the presence of a ϕ29-type DNA polymerase and a terminal protein (see below) of a ϕ29-type DNA polymerase.

By heterologous is meant any DNA which does not naturally occur within a ϕ29-type phage DNA molecule. This includes DNA encoding any desired protein.

A terminal DNA sequence is a sequence which naturally occurs at one or both ends of a ϕ29-type phage DNA which may be between 6 and 300 bases long. This sequence is specifically recognized and bound by a terminal protein, for example, the p3 protein of ϕ29-type.

In preferred embodiments, the method includes providing a ϕ29-type terminal DNA sequence at each end of the DNA molecule to be amplified; the terminal sequence is provided on a DNA fragment of less than 500 nucleotides; and the terminal protein is the terminal protein of the ϕ29-type phage in which the ϕ29-type DNA polymerase naturally occurs.

This invention provides a DNA polymerase which is highly processive, and may be produced with a low exonuclease activity. The high processivity of the polymerase makes it suitable, not only for DNA sequencing, but also for amplification of very large fragments of DNA (in excess of 10 kilobases in length). This makes the polymerase useful in a polymerase chain reaction (PCR)-type procedure or in replicative-type, protein primed, extension reactions. These long lengths of DNA are of use in forensic work, when small samples of DNA are available, and for restriction fragment length polymorphism analysis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The drawing will first briefly be described.

Drawing

The FIGURE is a represention of the amino acid sequence of various DNA polymerases showing sites of homology between the polymerases. ExoI, ExoII and ExoIII refer to the three regions of amino acid homology found amoung the different DNA polymerases compared in the FIGURE. Stars indicate the *E. coli* DNA polymerase I residues involved in either metal binding, or exonucleolytic catalysis. Asterisks indicate the *E. coli* DNA polymerase I residues involved in single-stranded DNA binding. Boxes shown by lines or arrows, and lettered or numbered, are α-helix and β-sheet regions respectively of DNA polymerase I.

DNA Polymerase

In general, a DNA polymerase of this invention is processive and has naturally-occurring exonuclease activity associated with it. In some preferred embodiments, the DNA polymerase has little or no associated exonuclease activity. These polymerases also have a strand-displacement activity.

By processive is meant that the DNA polymerase is able to continuously incorporate nucleotides using the same primer template, without dissociating from either or both the primer or the template molecules, under conditions normally used for DNA sequencing extension reactions, or other primer extension reactions. Generally, polymerases of the present invention will remain bound to the extended primer or template for at least 1–2 kilobases, generally at least 5 kb–10 kb, under suitable environmental conditions.

The ability of the polymerases of this invention to produce strand-displacement is advantageous in this invention because, in combination with high processivity, it allows synthesis of long DNA molecules of at least 70 kb, or even greater. Strand displacement activity is measured by any standard technique, for example, a polymerase may be incubated in a mixture with a single-stranded circular DNA molecule (e.g., M13) and a primer. If DNA molecules of length greater than the original circular molecule are synthesized, then the polymerase is able to displace DNA strands of a double-stranded molecule and continue to synthesize DNA—thus, it has a strand displacement activity. Such activity is generally present in a single protein molecule, e.g., p2 of ϕ29, and does not require energy in the form of ATP or its equivalent, utilizing only the standard deoxynucleoside triphosphates required to synthesize DNA. This activity is also observed when DNA synthesis is initiated by a terminal protein, e.g., p3 of ϕ29.

The exonuclease activity associated with DNA polymerases of this invention does not appear to significantly interfere with the use of the polymerase in a DNA sequencing reaction. However, it is preferred that the level of exonuclease activity be reduced to a level which is less than 10% or 1%, preferably less than 0.1% of the activity normally associated with DNA polymerases isolated from cells infected with naturally-occuring bacteriophage.

The DNA polymerases of this invention include polymerases which have been genetically modified to reduce the exonuclease activity of that polymerase, as well as those which are substantially identical to a naturally-occurring ϕ29-type DNA polymerase or a modified polymerase thereof, or to the equivalent enzymes enumerated above. Each of these enzymes can be modified to have properties similar to those of the ϕ29 DNA polymerase. It is possible to isolate the enzyme from phage-infected cells directly, but preferably the enzyme is isolated from cells which over-produce it.

By substantially identical is meant that the enzyme may contain amino acid substitutions which do not affect the overall properties of the enzyme. One example of a particularly desirable amino acid substitution is one in which the natural enzyme is modified to remove any exonuclease activity. This modification may be performed by genetic or chemical means.

As an example of this invention we shall describe the use of ϕ29 DNA polymerase in a variety of useful procedures. This example is not meant to be limiting to the invention; those skilled in the art will recognize that any of the above enumerated DNA polymerases can be similarly used in the manner described below.

ϕ29 DNA Polymerase

Bacteriophage ϕ29 is a linear double-stranded DNA molecule having a protein of 31 kD covalently linked at the 5′ end. This terminal protein, termed p3, is the product of viral gene 3, and is linked to the DNA by a phosphoester-bond between the OH group of a serine residue and 5′-dAMP. ϕ29 replication is initiated at either DNA end by a protein priming mechanism in which a free molecule of the terminal protein p3 reacts with dATP to form a protein-p3-dAMP covalent complex that provides the 3′ OH group needed for elongation. The initiation reaction requires, in addition to the gene 3 product and the ϕ29 DNA-protein p3 template, the product of the viral gene 2 (p2), which is the DNA polymerase. Protein p2 produced from gene 2 has a molecular weight of 66.5 kD. Associated with protein p2 is a 3′–5′ exonuclease activity active on single stranded and to some extent on double stranded DNA. Protein p2 may be purified by standard procedure from *E. coli* cells harboring a gene 2 containing recombinant plasmid, as described by Blanco et al., 29 Gene 33, 1984. The protein may be further purified by passage over a phosphocellulose column, as described by Blanco et al., 13 Nuc. Acid. Res. 1239, 1985. Blanco et al., id., also describe an exonuclease assay suitable for determination of inactivation of the exonuclease activity by genetic manipulation, as described below. Other enzymes associated with p2 and p3 in bacteriophage ϕ29 include p5 and p6, which increase the efficiency of polymerization by p2, as described by Salas, 109 Current Topics in Microbiology and Immunology 89, 1983.

Exonuclease Mutants

We shall now briefly describe the cloning of ϕ29 DNA polymerase and the manipulation of the p2 gene to produce examples of exonuclease mutants useful in this invention.

The starting plasmid was pBw2, which is a pBR322 derivative containing gene 2 of phage ϕ29, coding for the ϕ29 DNA polymerase, and including its ribosome-binding sequence (RBS) (Blanco et al. 29 Gene 33, 1984). In this construction the putative ATG initiation codon for the ϕ29 DNA polymerase is located 30 bp downstream a unique HindIII restriction site. Plasmid pBw2 was linearized with Hind III and subjected to a controlled digestion with the nuclease Bal31. The DNA was then digested with the restriction nuclease ScaI, which cuts 444 base pairs downstream gene 2, and the 5′ protruding ends were filled-in with the Klenow fragment of *E. coli* DNA polymerase I. The DNA fragment containing gene 2 was ligated with the T4 DNA ligase to plasmid pAZe3ss (Zaballos et al., 58 Gene 67, 1987) and digested with NcoI, whose 5′ protruding ends were then filled-in using Klenow fragment. The ligation product was used to transform competent *E. coli* M72 cells (lysogenic for bacteriophage λ and containing the temperature-sensitive cI857 repressor) and ampicillin-resistant bacteria selected. The latter were replica-plated in plates containing ampicillin (100 μ/ml) by growing them overnight at 30° C., followed by 3 h at 42° C. The colonies were transfered to nitrocellulose filters and lysed with 0.1% sodium dodecyl sulfate. The filters were washed, incubated with rabbit anti-ϕ29 DNA polymerase serum (produced by standard procedure) and the ϕ29 DNA polymerase-containing colonies were detected by incubation with [^{125}I] protein A followed by autoradiography. DNA sequencing of the selected clones allowed selection of the recombinant plasmids pAZw200 and pAZa203, which include ϕ29 DNA starting at the ATG triplets corresponding to position 2869–2867 and 2860–2858, respectively, in the open reading frame coding for p2, from the left ϕ29 DNA end (Yoshikawa et al., 17 Gene, 323, 1982). When the *E. coli* M72 cells, transformed with the recombinant plasmids pAZw200 or pAZa203, containing the gene coding for the ϕ29 DNA polymerase under the control of the P_L promoter of bacteriophage λ and with the RBS of gene ner of bacteriophage Mu, were grown at 30° C. and then shifted to 42° C. for 20 min to inactivate the λ CI857 repressor, followed by 2 h at 38° C., enzymatically active ϕ29 DNA polymerase was synthesized. About 150 and 300 μg of highly purified ϕ29 DNA polymerase was obtained per g of cells transformed with the recombinant plasmids pAZw200 and pAZa203, respectively.

The EcoRI-Hind III fragment from the recombinant plasmid pAZw200, containing the ϕ29 DNA polymerase gene and the RBS of gene ner of bacteriophage Mu was ligated, using T4 DNA ligase, to the EcoRI-HindIII sites of the replicative form of bacteriophage M13mp19. *E. coli* JM103 cells were transfected with such DNA and white plaques were selected in plates containing X-gal and isopropilthiogalactoside (IPTG). The selected plaques were amplified in liquid medium and the replicative form was isolated to check (by restriction analysis) the presence of the desired EcoRI-HindIII fragment. The single-stranded DNA was also isolated and used for site-directed mutagenesis, carried out as described by Nakamaya et al., 14 Nucl. Acids Res. 9679, 1986. The synthetic oligodeoxynucleotides used for the site-directed mutagenesis were:

5′AGTTGTGCCTTTGAGAC (1)

5′GACTTTGCGACAACTAC (2)

5′CTCAAATTTGCCGGAGC (3)

The recombinant clones containing point mutations were selected by hybridization to the corresponding mutagenic oligonucleotides 5′ [^{32}P]-labeled with T4 polynucleotide kinase and [λ−^{32}P] ATP. Single-stranded DNA was isolated from the selected clones and the sequence of the complete DNA polymerase gene was determined to check that each clone contained only the desired mutation. The EcoRI-BstBI fragment from the different clones was ligated with T4 DNA ligase to the same sites of plasmid pABw2, which contains the EcoRI-HindIII fragment of plasmid pAZw200 cloned at the corresponding sites of plasmid pT7-3 of the pT7 series (Tabor et al. 82 Proc. Natl. Acad. Sci. U.S.A., 1074, 1985), under the control of the

ϕ10 promoter of bacteriophage T7. This EcoRI-BstBI fragment replaces the wild-type sequence in that region by the corresponding mutant sequence. In this way, the recombinant plasmids pABn2D12A, pABn2E14A, pABn2D66A, pABn2D12AD66A and pABn2E1-4AD66A were selected, containing the corresponding amino acid changes from the amino-terminal end of the ϕ29 DNA polymerase. The recombinant plasmids were used to transform *E. coli* BL21 (DE3) cells containing the bacteriophage T7 RNA polymerase gene in the host DNA under the control of the lac uv5 promoter (Studier et al., 189 J. Mol. Biol. 113, 1986) being, therefore, inducible by IPTG. The ampicillin-resistant bacteria were analyzed for the presence of recombinant plasmids. Expression of the ϕ29 DNA polymerase mutant proteins was obtained by addition of 1 mM IPTG to *E. coli* cells containing the recombinant plasmids, grown at 37° C. and incubated for 1 h at 37° C. Five different mutant proteins were obtained, with the following amino acid changes: (1) alanine at position 12 (with reference to the first methionine in the gene encoding p2) in place of the natural asparatic acid (D12A); (2) alanine at position 14 instead of glutamic acid (E14A); (3) alanine at position 66 instead of aspartic acid (D66A); (4) alanine at positions 12 and 66 instead of aspartic acid (D12A, D66A); and (5) alanine at position 14 and 66 (E14A, D66A). The different mutant proteins were purified and their 3'–5' exonuclease activity determined by the above standard assay to be 100–1000 fold lower than that of the wild-type naturally occurring ϕ29 DNA polymerase.

Deposits

Strains pAZW200 (wild type p2 gene), pKC30Al (wild type p3 gene), pABN2D12AD66A (exonuclease deficient p2 gene having alanine at positions 12 and 66) have been deposited on Mar. 24, 1989, with the ATCC and assigned Numbers 67918, 67919, 67920 respectively.

Applicants and their assignees, Spanish Research Council (Consejo Superior De Investigaciones Scientificas, Serrano No. 117, 28006, Madrid, SPAIN), acknowledge their responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, 5 years after the last request for a culture, or 30 whichever is the longer, and its responsibility to notify the depository of the issuance of such a patent, at which time the deposits will be made irrevocably available to the public. Until that time the deposits will be made available to the Commissioner of Patents under the terms of 37 CFR Section 1–14 and 35 USC Section 112.

Referring to the Figure, the oligonucleotides used to form the above mutants were selected by taking into account the amino acid sequence homology with other polymerases and those mutations known to reduce exonuclease activity of DNA polymerase I. Derbyshire et al. 240 Science 199, 1988. Other mutations which are likely to produce suitable exonuclease mutants are shown in the black boxes. Generally, the amino acid at these portions is either deleted or replaced with a different amino acid. Large deletions or multiple replacement of amino acids are also useful in this invention. After mutagenesis, the level of exonuclease activity is measured and the amount of DNA polymerase activity determined to ensure it is sufficient for use in this invention (e.g., for DNA sequencing), being processive and having strand displacement activity.

Uses

DNA polymerases of this invention are useful in the following methods:

Filling in the 3' recessed termini created by digestion of DNA with restriction enzymes; labelling the termini of DNA fragments with protruding 5' ends (filling in reaction); labelling the termini of blunt-ended DNA fragments or DNA fragments with protruding 3' termini (exchange reaction); removing the 3' protruding termini of DNA fragments; labelling DNA fragments for use as hybridization probes by partial digestion of double-stranded DNA using the naturally associated 3'–5' exonuclease activity, followed by a filling reaction with labelled dNTPs (displacement reaction); synthesis of long (over 5–10 kb) as well as short single-stranded DNA probes containing multiple copies of a desired sequence, obtained by strand-displacement synthesis on single stranded DNA, such long probes may be labelled with labelled dNTPs at a high specific activity; random labelling of double-stranded DNA at a high specific activity by using degenerated oligonucleotide primers; second-strand cDNA synthesis in cDNA cloning; sequencing DNA using a Sanger-type dideoxy system (Sanger et al. 74 Proc. Natl. Acad. Sci. U.S.A. 5463, 1977) on single- and double-stranded DNA templates; sequencing DNA by a plus/minus-type method (Sanger et al., 94 J. Mol. Biol. 441, 1975); random mutagenesis of single- and double-stranded DNA templates by using an exonuclease-deficient DNA polymerase under conditions of low DNA replication fidelity; site-directed mutagenesis on double stranded DNA templates; gene amplification or synthesis of long double-stranded DNA fragments using synthetic oligonucleotides as primers; and amplification or synthesis of double-stranded DNA fragments using a ϕ29-type DNA replication system including a ϕ29-type DNA polymerase, a terminal protein, any accessory proteins necessary to enhance the reaction, and a ϕ29-type DNA-protein p3 template.

The ϕ29-type DNA polymerases are particularly useful for DNA sequencing, performing a polymerase chain reaction, and for amplification without the need for temperature cycling to produce extremely long strands of DNA. These methods will now be discussed in detail.

EXAMPLE 1

P.C.R.

There follows an example of a polymerase chain reaction using ϕ29 DNA polymerase. In general, the DNA polymerase may simply be used in place of Klenow or Taq polymerases.

0.1 pmol of target DNA are mixed with 300 pmol each of selected oligonucleotides (15–20 mers), and 75 nmol of each deoxynucleoside triphosphate (1N5 mM) in 50 μl of a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM magnesium chloride. The solution is brought to 95° C. for 10 minutes, and cooled to 30° C. for 1 min in a waterbath. 1 μl containing 20 ng of ϕ29 DNA polymerase (either wild type or an exonuclease mutant) is added to the mixture and the reaction allowed to proceed for 5 min at 30° C., after which the cycle of heating, cooling, adding enzyme, and reacting is repeated about nine times. The polymerase used is purified by standard procedures.

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Prior polymerases used in polymerase chain reactions failed to provide DNA fragments in the size range greater than about 2 kilobases (Saiki et al., 239 Science 487, 1988; Keohavong et al., 71 Gene 211, 1988). This relatively short size is probably due to the secondary structure and hinderance produced by reannealing of the DNA fragment, which impedes the progress of these DNA polymerases. Because ϕ29 DNA polymerase has a high processivity and strand displacement ability, it is an ideal enzyme for DNA amplification to produce long amplified molecules.

EXAMPLE 2

DNA Sequencing

For DNA sequencing, the sequence procedure using single-stranded DNA as a template was essentially as described by Tabor et al., 84 Proc. Natl. Acad. Sci. U.S.A. 4767, 1987 with some modification.

In the annealing reaction, the annealing reaction mixture (20 μl) contained 2.5 μg of template DNA, 60 ng of primer (a 10-fold molar ratio to the template) in a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 75 mM NaCl. The mixture was heated to 65° C. for 15 min and then cooled to room temperature (20°–25° C.) over 30 min.

In the labelling reaction, a single labelling reaction was used for all four termination reactions. To the annealing mixture (20 μl) was added 20 μl of a mixture containing 0.6 μM each dGTP, dTTP, dCTP and [α-^{32}P]dATP, 2 mM dithiothreitol, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 8% glycerol. Labelling was started by addition of the ϕ29 DNA polymerase (either wild type or exonuclease deficient, 150 ng). Incubation was at room temperature for 5 min, at which time the reaction was complete. Four aliquots (8 μl each) of the "labelling" reaction mixture were used for the "termination" reactions.

In the extension-termination reaction, four separate dideoxy "termination" mixtures were prepared in 1.0 ml microcentrifuge tubes. Each mixture (2 μl) contained 20 μM each of the three dNTPs, the remaining dNTP, and its corresponding dideoxy- NTP, being at 2 μM and 200 μM, respectively. Eight microliters of the above labelling reaction mixture was added to each termination mixture and incubated for 15 min at 30° C. Three microliters of stop solution (95% formamide/20 mM EDTA/0.05% xylene cyanol/0.05% bromophenol blue) was then added. The mixtures were heated at 95° C. for 2 min immediately prior to loading 6 μl onto a sequencing gel.

A protocol for double-stranded DNA sequence is similar to the above protocol but preceded by an alkali-denaturation step.

For the reasons discussed above, the secondary structure of the DNA template may impede the progress of DNA polymerases. This may occur either at a pallindromic sequence, which may form a hairpin structure, or at other sequences where an enzyme pauses dependent upon a specific sequence. Because of the high processivity and strand displacement ability of ϕ29 DNA polymerases, the sequencing results obtained with this polymerase are superior to those of the prior art.

In the following methods it is useful to include accessory proteins, such as p5 and p6 in the reaction mix. Preparation of p6 is described by Blanco et al., 62 J. Virol. 4167, 1988. Preparation of p5 was as follows:

E. coli K12ΔH1Δtrp cells carrying the gene 5-containing recombinant plasmid pGM26 or *B. subtilis* cells infected with the phage ϕ29 mutant sus 14(1242) were used as a source of protein p5 for purification. The protein p5 present in the *E. coli* extracts amounted to ~1.4% of the total protein after 2.5 h of induction at 42° C., and that present in the *B. subtilis* extracts was ~2.7% of the total protein.

Ten g of *E. coli* K12ΔH1Δtrp cells harboring the gene 5-containing recombinant plasmid pGM26, were induced for 2.5 h at 42° C., were ground with alumina (20 g) and extracted with buffer A (50 mM Tris-HCl, pH 7.5 5% glycerol) containing 0.3M KCl. The lysate was centrifuged for 10 min at 16,500×g and the pellet reextracted with the same buffer. The two supernatants were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was dissolved in buffer A, dialyzed against the same buffer, diluted with buffer A +20% glycerol and passed through a DEAE- cellulose column (2.7 cm×10 cm) equilibrated with buffer A+10 mM NaCl. THe column was washed first with buffer A+20% glycerol, then with buffer A, and protein p5 was finally eluted with buffer A+50 mM NaCl. The fractions containing protein p5 were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was resuspended in 1.2 ml of buffer A+1.4M ammonium sulfate and 50% glycerol. The pellet remaining after centrifugation, containing most of protein p5, was dissolved in buffer A+50% glycerol. Protein p5 was purified by a similar procedure from *B. subtilis* cells infected with the ϕ29 delayed lysis mutant sus14(1242). In all purification steps protein p5 was followed by SDS-polyacrylamide gel electrophoresis.

In some preparations, after the last purification step, protein p5 was centrifuged for 24 h at 260,000×g at 0° C. in a 5 ml 15 to 30% (v/v) glycerol gradient in 50 mM Tris-HCl, pH 7.5, 0.2M NaCl. After centrifugation, 0.2 ml fractions were collected and the presence of protein p5 was determined.

EXAMPLE 3

Replicative-type Amplification

As described above, long strands of DNA may be synthesized by primer elongation using a ϕ29-type DNA polymerase. This property may be used to amplify DNA without the need for the temperature cycling used in prior polymerase chain reactions. This process makes use of a protein primer rather than an oligonucleotide primer. Generally, the terminal repeat sequences of a ϕ29-type DNA polymerase are covalently bonded by any of a number of standard methods, to each end of the DNA molecule to be amplified. This bonding may be by direct ligation of the sequence to be amplified, or may be by a procedure similar to site-directed mutagenesis, where an oligonucleotide comprising the terminal sequence is constructed to allow recombination of that nucleotide sequence to adjacent DNA which is required to be amplified. In an alternative method, a restriction endonuclease may be used to randomly cut genomic DNA and synthetic oligonucleotides, comprising the terminal sequences, placed at these sites. In each case the DNA is amplified by provision of p2 and p3 proteins, along with nucleoside triphosphates. An example of this now follows:

A preparation of ϕ29 DNA protein-p3 isolated by standard procedure is cut with the restriction nuclease ClaI to produce two fragments of length 6147 and 13138 bp. A DNA fragment containing an appropriate multicloning site is then ligated to join the two ClaI

fragments. The DNA fragment to be amplified is then ligated to one of the multicloning sites and the resulting DNA is used as a template for DNA synthesis.

The incubation mixture contains, in 25 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM spermidine, 20 mM ammonium sulfate, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP, 80 ng of φ29 DNA polymerase, 20 ng of terminal protein p3 purified as described (Prieto et al. 81 Proc. Natl. Acad. Sci. U.S.A., 1639, 1984) from *E. coli* N99λts cells harboring the gene 3-containing recombinant plasmid pKC30Al (Garcia et al., 21 Gene 65, 1983), proteins p5 (9 μg) and p6 (2 μg) purified from φ29-infected *B. subtilis* as described above, and the desired amount of template (10 ng–1 μg). After incubation for 60 min. at 30° C., 25 μl containing all the components of the system except the template are added and the mixture is incubated again for 60 min. at 30° C. (2nd cycle). The cycles are repeated in the same way several times.

EXAMPLE 4

Synthesis of Long Strands of DNA

The DNA polymerases of this invention permit ready synthesis of very long DNA molecules useful in a large number of applications, e.g., RFLP analysis, and DNA probe construction. There follows an example of this methodology.

Single-stranded M13 DNA was hybridized with a 17-mer M13 oligonucleotide primer. The incubation mixture contained, in 10 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.5 μg of primed M13 DNA, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P] dATP and φ29 DNA polymerase (50 ng). After incubation for 40 min at 30° C. the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% sodium dodecyl sulfate and the Cerenkov radiation of the excluded fraction was counted. To analyze the size of the DNA synthesized, a sample was subjected to electrophoresis in alkaline 0.7% agarose gels along with DNA length markers. The DNA markers were detected with ethidium bromide and the synthesized DNA was detected by autoradiography of the dried gel. In 40 min of incubation at 30° C., DNA longer than 70 Kb was synthesized.

Other embodiments are within the following claims.

We claim:

1. In a method for determining the nucleotide base sequence of a DNA molecule, comprising the steps of:

annealing said DNA molecule with a primer molecule able to hybridize to said DNA molecule;

incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each said agent terminates DNA synthesis at a different nucleotide base; and

separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of said DNA can be determined,

the improvement wherein said DNA polymerase comprises a φ29 type DNA polymerase or an exonuclease deficient φ29-type DNA polymerase.

2. The method of claim 1 wherein said φ29-type DNA polymerase is that phage DNA polymerase in cells infected with a φ29-type phage.

3. The method of claim 1 wherein said φ29-type DNA polymerase is selected from the DNA polymerases of the phage group consisting of: φ29, Cp-1, PRD1, φ15, φ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17.

4. The method of claim 1 wherein said φ29-type DNA polymease is a modified polymerase having less than ten percent of the exonuclease activity of the naturally-occurring polymerase.

5. The method of claim 4 wherein said polymerase is modified to have less than one percent of the naturally-occurring exonuclease activity.

6. The method of claim 5 wherein said φ29-type DNA polymerase has substantially no exonuclease activity.

7. The method of claim 1 wherein said terminating agent is a dideoxynucleoside triphosphate.

8. A kit for DNA sequencing, comprising:

a φ29-type DNA polymerase or an exonuclease deficient φ29-type DNA polymerase, and a chain terminating agent.

9. In a method for amplification of a DNA sequence comprising annealing a first and second primer to opposite strands of a double-stranded DNA sequence and incubating the annealed mixture with a DNA polymerase,

the improvement comprising employing as said DNA polymerase a φ29-type DNA polymerase or an exonuclease deficient φ29-type DNA polymerase.

10. The method of claim 9 wherein said first and second primers have their 3' ends directed towards each other after annealing.

11. The method of claim 9 wherein said method further comprises, after said incubation step, denaturing the resulting DNA, annealing said first and second primers to the denatured DNA and incubating the last said annealed mixture with said polymerase.

12. The method of claim 11 wherein said cycle of denaturing, annealing, and incubating is repeated from 10–40 times.

13. The method of claim 9 wherein said φ29-type DNA polymerase is selected from the DNA polymerases of the phage group consisting of: φ29, Cp-1, PRD1, φ15, φ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17.

14. The method of claim 9 wherein said polymerase exhibits less than 10% of the naturally-occurring exonuclease activity exhibited by the corresponding naturally-occurring polymerase.

15. The method of claim 9 wherein said DNA polymerase has no detectable exonuclease activity.

16. A method for production of DNA molecules of greater than 10 kilobases in length comprising:

providing a template DNA molecule;

annealing a primer with said template molecule; and

incubating the annealed primer and template molecules in the presence of a φ29-type DNA polymerase or an exonuclease deficient φ29-type DNA polymerase and a mixture of four different deoxynucleoside triphosphates.

17. A method for amplification of a heterologous DNA molecule comprising the steps of:

covalently bonding a φ29-type terminal DNA sequence at one end of said DNA molecule to form a product; and

incubating said product in the presence of a φ29-type DNA polymerase, a terminal protein of a φ29-type DNA polymerase or an exonuclease deficient φ29-

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type DNA polymerase and a mixture of four different deoxynucleoside triphosphates.

18. The method of claim 17 further comprising the step of providing a ϕ29-type terminal sequence at each end of said DNA molecule.

19. The method of claim 18 wherein said terminal sequence is provided on a DNA fragment of less than 500 nucleotides.

20. The method of claim 17 wherein said terminal protein is the terminal protein of the ϕ29-type phage in which the ϕ29-type DNA polymerase naturally occurs.

* * * * *

US005198543A

United States Patent [19]

Blanco et al.

[11] **Patent Number: 5,198,543**

[45] **Date of Patent: Mar. 30, 1993**

[54] **PHI29 DNA POLYMERASE**

[75] Inventors: **Luis Blanco; Antonio Bernad; Margarita Salas,** all of Madrid, Spain

[73] Assignee: **Consejo Superior Investigaciones Cientificas,** Madrid, Spain

[21] Appl. No.: **668,945**

[22] Filed: **Mar. 13, 1991**

[51] **Int. Cl.**[5] **C07H 15/12**

[52] **U.S. Cl.** **536/23.2**; 536/23.72; 435/6

[58] **Field of Search** 435/194; 536/26, 27, 536/28, 29

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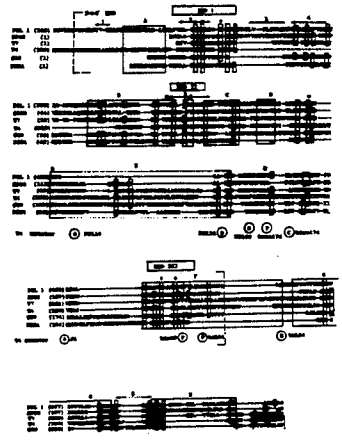
Assistant Examiner—Stephanie W. Zitomer
Attorney, Agent, or Firm—Lyon & Lyon

[57] **ABSTRACT**

An improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is provision of a DNA polymerase which is a ϕ29-type DNA polymerase.

3 Claims, 2 Drawing Sheets



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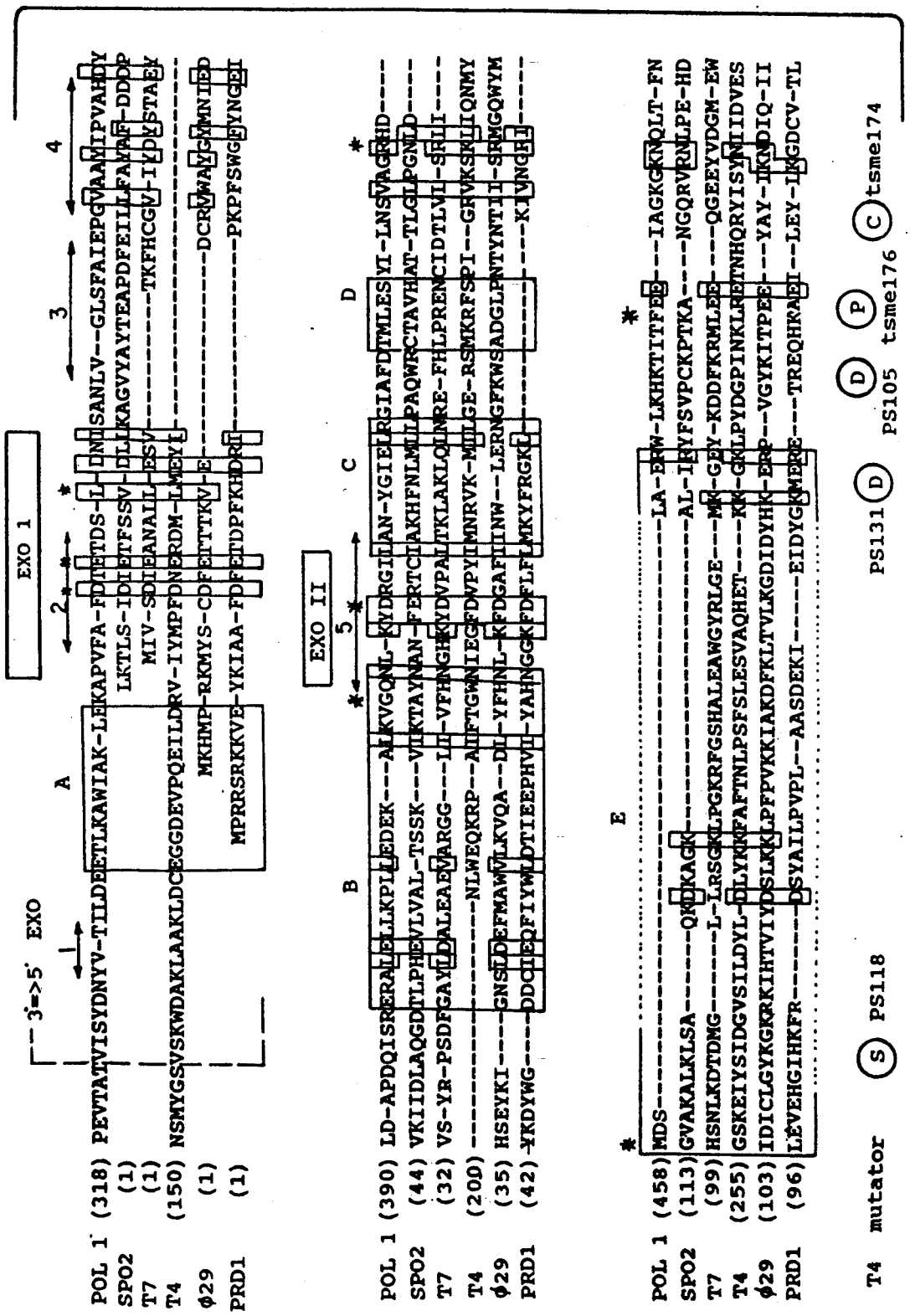
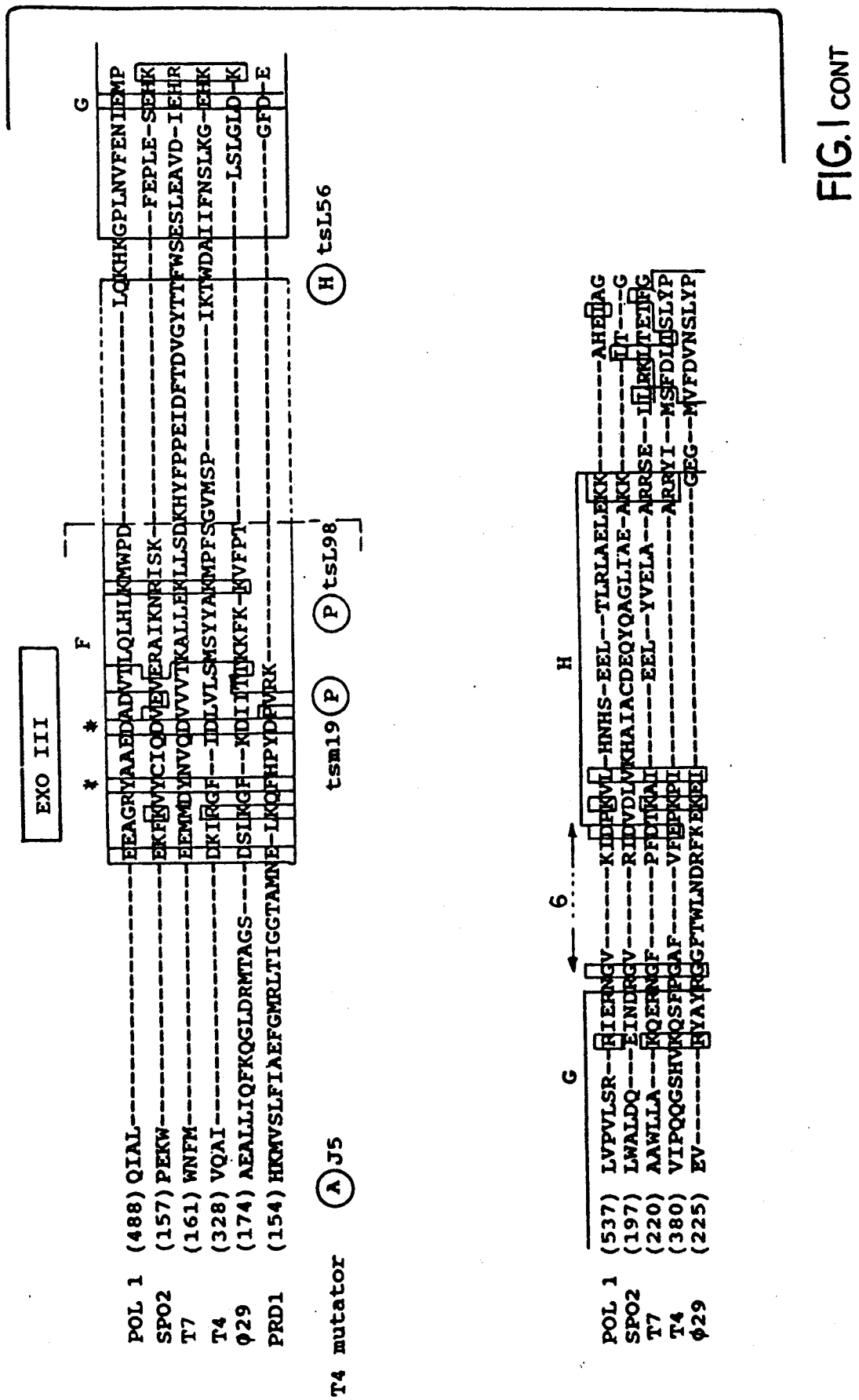

3'=>5' EXO
EXO 1
EXO II
A
B
C
D
E
1
2
3
4
5
POL 1 (318) PEVTATVISYDNYV-TILDEETLKAWIAK-LEKAPVFA-FDTETDS-L-DNLSANLV--GLSFAIEPGVAAMIPVAHDY
SPO2 (1) LKTLS-IDIETFSSV-DLLKAGVYAYTEAPDFEIILFAVAF-DDDP
T7 (1) MIV-SDIEANAIL-ESV----------TKFHCGVI-IYDLPSTAEY
T4 (150) NSMYGSVSKWDAKLAAKLDCEGGDEVPQEILDRV-IYMPFDNERDM-LMEYL----------DCRVWALGIMNIED
ϕ29 (1) MKHMP-RKMYS-CDFETTTKV-E----------PKPFSWGFVNGEI
PRD1 (1) MPRRSRKKVE-YKIAA-FDFETDPFKHDRI----------
POL 1 (390) LD-APDQISRERALELLKPLEDEK---AFKVGQNL-KYDRGIIAN-YGIELRGIAFDIMLESYI-LNSVAGRHD----
SPO2 (44) VKIIDLAQGDTLPHEVLVAL-TSSK---VIKKTAYNAN-FERTCIAKHFNLMLLPAQWRCTAVHAT-TLGLPGNLD----
T7 (32) VS-YR-PSDFGAYLDALEAFVARGG---LI-VFHNGHKYDVPALTKLAKLQLNRE-FHLPRENCIDTLVL-SRLI----
T4 (200) ----------NLWEQKRP--AIFTGWNIEGFDVPYIMNRVK-MLLGE-RSMKRFSPI--GRVKSKLIQNMY
ϕ29 (35) HSEYKI----GNSLDEFMAWVLKVQA--DL-YFHNL-KFDGAFIINW--LERNGFKWSADGLPNTYNTII-SRMGQWYM
PRD1 (42) -YKDYWG----DDCIEDFIYWLDTIEEPHVI-YAHNGGKFDFLFLMKYFRGKL----------KIVNGHI-----
POL 1 (458) MDS----------LA-EFW-LKHKTITFEE--IAGKGKNQLT-FN
SPO2 (113) GVAKALKLSA----------QKDKAGK----------AL-IRYFSVPCKPTKA----NGQRVRNLPE-HD
T7 (99) HSNLKDTDMG----------L-LRSGKLPGKRFGSHALEAWGYRLGE----MK-GEY-KDDFKRMLEE----QGEEYVDGM-EW
T4 (255) GSKEIYSIDGVSILDYL-DLYKKFAFTNLPSFSLESVAQHET----KM-GKLPYDGPINKLRETNHQRYISYNIIDVES
ϕ29 (103) IDICLGYKQKRKIHTVIYDSLKKLPFPVKKIAKDFKLTVLKGDIDYHK-ERP--VGYKITPEE--YAY-IKNDIQ-II
PRD1 (96) LEVEHGIHKFR----------DSYAILPVPL--AASDEKI----EIDYGKMERE---TREQHKAEI--LEY-LKGDCV-TL
T4 mutator S PS118 PS131 D PS105 tsme176 D P C tsme174

FIG. 1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φ29 (174) AEALLIQFKQGLDRMTAGS
PRD1 (154) HKMVSLFIAEFGMRLTIGGTAMN
T4 mutator
A J5
tsm19 P
P tsL98
H tsL56
POL 1 (537) LVPVLSR
SPO2 (197) LWALDQ
T7 (220) AAWLLA
T4 (380) VIPQQGSHVAQSFPGAF
φ29 (225) EV
G
H
6

FIG. 1 CONT

PHI29 DNA POLYMERASE

This is a divisional application(s) Ser. No. 07/328,462 filed Mar. 24, 1989, now U.S. Pat. No. 5,001,050, issued Mar. 19, 1991.

BACKGROUND OF THE INVENTION

This invention relates to DNA polymerases suitable for DNA sequencing.

DNA sequencing involves the generation of four populations of single-stranded DNA fragments, having one defined terminus and one variable terminus. The variable terminus always terminates at a specific given nucleotide base (either guanine (G), adenine (A), thymine (T), or cytosine (C)). The four different sets of fragments are each separated on the basis of their length, on a high resolution polyacrylamide gel; each band on the gel corresponds colinearly to a specific nucleotide in the DNA sequence, thus identifying the positions in the sequence of the given nucleotide base.

Generally there are two methods of DNA sequencing. One method (Maxam and Gilbert sequencing) involves the chemical degradation of isolated DNA fragments, each labeled with a single radiolabel at its defined terminus, each reaction yielding a limited cleavage specifically at one or more of the four bases (G, A, T or C). The other method (dideoxy sequencing) involves the enzymatic synthesis of a DNA strand. Four separate syntheses are run, each reaction being caused to terminate at a specific base (G, A, T or C) via incorporation of the appropriate chain terminating dideoxynucleotide. The latter method is preferred since the DNA fragments are uniformly labelled (instead of end labelled) and thus the larger DNA fragments contain increasingly more radioactivity. Further, ^{35}S labelled nucleotides can be used in place of ^{32}P labelled nucleotides, resulting in sharper definition; and the reaction products are simple to interpret since each lane corresponds only to either G, A, T or C. The enzymes used for most dideoxy sequencing is the *Escherichia coli* DNA-polymerase I large fragment ("Klenow"), AMV reverse transcriptase, and T7 DNA polymerase (Tabor et al., U.S. Pat. No. 4,795,699). The T7 DNA polymerase used for sequencing is said to be advantageous over other DNA polymerases because it is processive, has no associated exonuclease activity, does not discriminate against nucleotide analog incorporation, and can utilize small oligonucleotides as primers. These properties are said to make the polymerase ideal for DNA sequencing. Id.

SUMMARY OF THE INVENTION

In a first aspect, the invention features an improved method for determining the nucleotide base sequence of a DNA molecule. The method includes annealing the DNA molecule with a primer molecule able to hybridize to the DNA molecule; incubating the annealed mixture in a vessel containing four different deoxynucleoside triphosphates, a DNA polymerase, and one or more DNA synthesis terminating agents which terminate DNA synthesis at a specific nucleotide base, wherein each the agent terminates DNA synthesis at a different nucleotide base; and separating the DNA products of the incubating reaction according to size, whereby at least a part of the nucleotide base sequence of the DNA can be determined. The improvement is

provision of a DNA polymerase which is a ϕ29-type DNA polymerase.

By ϕ29-type DNA polymerase is meant any DNA polymerase isolated from the related phages which contain a terminal protein used in the initiation of replication of DNA. These phages are generally described by Salas, 1 The Bacteriophages 169, 1988. These phages are closely related in the structure of their DNA polymerases, some differing by as few as 6 amino acid changes with 5 of those amino acids being replaced by similar amino acids. These phages have a short inverted terminal repeat sequence of length between about 6 and 300 nucleotides. These polymerases have a highly active 3'-5' exonuclease activity, but no 5'-3' exonuclease activity. Surprisingly, although they are related to the T4 family of DNA polymerases, they are able to adequately recognize chain terminating agents such as dideoxynucleosides and therefore are useful for DNA sequencing. This ability is even more surprising since the exonuclease is known to recognize both deoxy and dideoxy ADP. Blanco et al. 13 Nuc. Acid. Res. 1239, 1246, 1985.

In preferred embodiments, the ϕ29-type DNA polymerase is either that phage polymerase in cells infected with a ϕ29-type phage; the ϕ29-type DNA polymerase is chosen from the DNA polymerases of phases ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; or the DNA polymerase is a ϕ29-type polymerase, modified to have less than ten percent of the exonuclease activity of the naturally-occurring polymerase, most preferably the polymerase has less than one percent, and even more preferably has substantially no exonuclease activity; and the terminating agent is a dideoxynucleotide.

In a related aspect, the invention features a kit for DNA sequencing including a supply of ϕ29-type DNA polymerase, together with a supply of a chain terminating agent. By kit is meant a container designed to keep these two components separated from each other, preferably in condition for use in a DNA sequencing reaction.

In another related aspect, the invention features a DNA fragment encoding a modified ϕ29-type DNA polymerase, wherein the polymerase has sufficient DNA polymerase activity for use in DNA sequencing, and an exonuclease activity which is less than 10% the activity of the corresponding naturally occurring ϕ29-type DNA polymerase.

By corresponding is meant that the modified polymerase is derived from a naturally occurring polymerase, generally by in vitro mutagenesis of the DNA sequence encoding the latter polymerase, and the latter is the corresponding polymerase.

In preferred embodiments, the DNA fragment is modified to substantially eliminate the naturally-occurring exonuclease activity; and the DNA fragment includes a DNA sequence encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14, or 16 of the polymerase is replaced by an alanine moiety.

The invention also features a ϕ29-type DNA polymerase produced from the above described DNA fragments.

In another aspect, the invention features an improved method for amplification of a DNA sequence. The method includes annealing a first and second primer to opposite strands of a double stranded DNA sequence, and incubating the annealed mixture with a DNA poly-

merase. The improvement includes employing as the DNA polymerase a ϕ29 type DNA polymerase.

In preferred embodiments, the first and second primers have their 3′ ends directed towards each other after annealing; the method further includes, after the incubation step, denaturing the resulting DNA, annealing the first and second primers to the denatured DNA and incubating the last annealed mixture with the polymerase; the cycle of denaturing, annealing, and incubating is repeated from 10–40 times; the ϕ29-type DNA polymerase is selected from the DNA polymerases of phages. ϕ29, Cp-1, PRD1, ϕ15, ϕ21, PZE, PZA, Nf, M2Y, B103, SF5, GA-1, Cp-5, Cp-7, PR4, PR5, PR722, and L17; the DNA polymerase exhibits less than 10% of the naturally-occurring exonuclease activity exhibited by the corresponding naturally-occurring polymerase, most preferably the polymerase has no detectable exonuclease activity.

In a further aspect, the invention features a method for production of DNA molecules of greater than 10 kilobases in length. The method includes providing a template DNA molecule; annealing a primer with the template molecule; and incubating the annealed primer and template molecules in the presence of a ϕ29-type DNA polymerase, and a mixture of four different deoxynucleoside triphosphates.

The invention also features a method for amplification of a heterologous DNA molecule including covalently bonding a ϕ29-type terminal DNA sequence at one end of the DNA molecule to form a product; and incubating the product in the presence of a ϕ29-type DNA polymerase and a terminal protein (see below) of a ϕ29-type DNA polymerase.

By heterologous is meant any DNA which does not naturally occur within a ϕ29-type phage DNA molecule. This includes DNA encoding any desired protein.

A terminal DNA sequence is a sequence which naturally occurs at one or both ends of a ϕ29-type phage DNA which may be between 6 and 300 bases long. This sequence is specifically recognized and bound by a terminal protein, for example, the p3 protein of ϕ29.

In preferred embodiments, the method includes providing a ϕ29-type terminal DNA sequence at each end of the DNA molecule to be amplified; the terminal sequence is provided on a DNA fragment of less than 500 nucleotides; and the terminal protein is the terminal protein of the ϕ29-type phage in which the ϕ29-type DNA polymerase naturally occurs.

This invention provides a DNA polymerase which is highly processive, and may be produced with a low exonuclease activity. The high processivity of the polymerase makes it suitable, not only for DNA sequencing, but also for amplification of very large fragments of DNA (in excess of 10 kilobases in length). This makes the polymerase useful in a polymerase chain reaction (PCR) type procedure or in replicative type, protein primed, extension reactions. These long lengths of DNA are of use in forensic work, when small samples of DNA are available, and for restriction fragment length polymorphism analysis.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The drawing will first briefly be described.

DRAWING

The FIGURE is a represention of the amino acid sequence of various DNA polymerases showing sites of homology between the polymerases.

ExoI, ExoII and ExoIII refer to the three regions of amino acid homology found among the different DNA polymerases compared in the FIGURE. Stars indicate the *E. coli* DNA polymerase I residues involved in either metal binding, or exonucleolytic catalysis. Asterisks indicate the *E. coli* DNA polymerase I residues involved in single-stranded DNA binding. Boxes shown by lines or arrows, and lettered or numbered, are α-helix and β-sheet regions respectively of DNA polymerase I.

DNA Polymerase

In general, a DNA polymerase of this invention is processive and has naturally-occurring exonuclease activity associated with it. In some preferred embodiments, the DNA polymerase has little or no associated exonuclease activity. These polymerases also have a strand displacement activity.

By processive is meant that the DNA polymerase is able to continuously incorporate nucleotides using the same primer template, without dissociating from either or both the primer or the template molecules, under conditions normally used for DNA sequencing extension reactions, or other primer extension reactions. Generally, polymerases of the present invention will remain bound to the extended primer or template for at least 1–2 kilobases, generally at least 5 kb–10 kb, under suitable environmental conditions.

The ability of the polymerases of this invention to produce strand-displacement is advantageous in this invention because, in combination with high processivity, it allows synthesis of long DNA molecules of at least 70 kb, or even greater. Strand displacement activity is measured by any standard technique, for example, a polymerase may be incubated in a mixture with a single-stranded circular DNA molecule (e.g., M13) and a primer. If DNA molecules of length greater than the original circular molecule are synthesized, then the polymerase is able to displace DNA strands of a double stranded molecule and continue to synthesize DNA—thus, it has a strand displacement activity. Such activity is generally present in a single protein molecule, e.g., p2 of ϕ29, and does not require energy in the form of ATP or its equivalent, utilizing only the standard deoxynucleoside triphosphates required to synthesize DNA. This activity is also observed when DNA synthesis is initiated by a terminal protein, e.g., p3 of ϕ29.

The exonuclease activity associated with DNA polymerases of this invention does not appear to significantly interfere with the use of the polymerase in a DNA sequencing reaction. However, it is preferred that the level of exonuclease activity be reduced to a level which is less than 10% or 10%, preferably less than 0.1% of the activity normally associated with DNA polymerases isolated from cells infected with naturally-occurring bacteriophage.

The DNA polymerases of this invention include polymerases which have been genetically modified to reduce the exonuclease activity of that polymerase, as well as those which are substantially identical to a naturally-occurring ϕ29-type DNA polymerase or a modified polymerase thereof, or to the equivalent enzymes enumerated above. Each of these enzymes can be modi-

fied to have properties similar to those of the ϕ29 DNA polymerase. It is possible to isolate the enzyme from phage-infected cells directly, but preferably the enzyme is isolated from cells which over-produce it.

By substantially identical is meant that the enzyme may contain amino acid substitutions which do not affect the overall properties of the enzyme. One example of a particularly desirable amino acid substitution is one in which the natural enzyme is modified to remove any exonuclease activity. This modification may be performed by genetic or chemical means.

As an example of this invention we shall describe the use of ϕ29 DNA polymerase in a variety of useful procedures. This example is not meant to be limiting to the invention; those skilled in the art will recognize that any of the above enumerated DNA polymerases can be similarly used in the manner described below.

ϕ29 DNA Polymerase

Bacteriophage ϕ29 is a linear double-stranded DNA molecule having a protein of 31 kD covalently linked at the 5′ end. This terminal protein, termed p3, is the product of viral gene 3, and is linked to the DNA by a phosphoester-bond between the OH group of a serine residue and 5′-dAMP. ϕ29 replication is initiated at either DNA end by a protein priming mechanism in which a free molecule of the terminal protein p3 reacts with dATP to form a protein-p3-dAMP covalent complex that provides the 3′ OH group needed for elongation. The initiation reaction requires, in addition to the gene 3 product and the ϕ29 DNA protein p3 template, the product of the viral gene 2 (p2), which is the DNA polymerase. Protein p2 produced from gene 2 has a molecular weight of 66.5 kD. Associated with protein p2 is a 3′-5′ exonuclease activity active on single stranded and to some extent on double stranded DNA. Protein p2 may be purified by standard procedure from *E. coli* cells harboring a gene 2 containing recombinant plasmid, as described by Blanco et al., 29 Gene 33, 1984. The protein may be further purified by passage over a phosphocellulose column, as described by Blanco et al., 13 Nuc. Acid. Res. 1239, 1985. Blanco et al., id., also describe an exonuclease assay suitable for determination of inactivation of the exonuclease activity by genetic manipulation, as described below. Other enzymes associated with p2 and p3 in bacteriophage ϕ29 include p5 and p6, which increase the efficiency of polymerization by p2, as described by Salas, 109 Current Topics in Microbiology and Immunology 89, 1983.

Exonuclease Mutants

We shall now briefly describe the cloning of ϕ29 DNA polymerase and the manipulation of the p2 gene to produce examples of exonuclease mutants useful in this invention.

The starting plasmid was pBw2, which is a pBR322 derivative containing gene 2 of phage ϕ29, coding for the ϕ29 DNA polymerase, and including its ribosome binding sequence (RBS) (Blanco et al. 29 Gene 33, 1984). In this construction the putative ATG initiation codon for the ϕ29 DNA polymerase is located 30 bp downstream a unique HindIII restriction site. Plasmid pBw2 was linearized with Hind III and subjected to a controlled digestion with the nuclease Bal31. The DNA was then digested with the restriction nuclease ScaI, which cuts 444 base pairs downstream gene 2, and the 5′ protruding ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I. The DNA fragment containing gene 2 was ligated with the T4 DNA ligase to plasmid pAZe3ss (Zaballos et al., 58 Gene 67, 1987) and digested with NcoI, whose 5′ protruding ends were then filled in using Klenow fragment. The ligation product was used to transform competent *E. coli* M72 cells (lysogenic for bacteriophage λ and containing the temperature sensitive cI857 repressor) and ampicillin resistant bacteria selected. The latter were replica-plated in plates containing ampicillin (100 μ/ml) by growing them overnight at 30° C., followed by 3 h at 42° C. The colonies were transfered to nitrocellulose filters and lysed with 0.1% sodium dodecyl sulfate. The filters were washed, incubated with rabbit anti ϕ29 DNA polymerase serum (produced by standard procedure) and the ϕ29 DNA polymerase-containing colonies were detected by incubation with [125I] protein A followed by autoradiography. DNA sequencing of the selected clones allowed selection of the recombinant plasmids pAZw200 and pAZa203, which include ϕ29 DNA starting at the ATG triplets corresponding to position 2869–2867 and 2860–2858, respectively, in the open reading frame coding for p2, from the left ϕ29 DNA end (Yoshikawa et al., 17 Gene, 323, 1982). When the *E. coli* M72 cells, transformed with the recombinant plasmids pAZw200 or pAZa203, containing the gene coding for the ϕ29 DNA polymerase under the control of the P_L promoter of bacteriophage λ and with the RBS of gene ner of bacteriophage Mu, were grown at 30° C. and then shifted to 42° C. for 20 min to inactivate the λ CI857 repressor, followed by 2 h at 38° C., enzymatically active ϕ29 DNA polymerase was synthesized. About 150 and 300 μg of highly purified ϕ29 DNA polymerase was obtained per g of cells transformed with the recombinant plasmids pAZw200 and pAZa203, respectively.

The EcoRI-Hind III fragment from the recombinant plasmid pAZw200, containing the ϕ29 DNA polymerase gene and the RBS of gene ner of bacteriophage Mu was ligated, using T4 DNA ligase, to the EcoRI HindIII sites of the replicative form of bacteriophage M13mp19. *E. coli* JM103 cells were transfected with such DNA and white plaques were selected in plates containing X-gal and isopropilthiogalactoside (IPTG). The selected plaques were amplified in liquid medium and the replicative form was isolated to check (by restriction analysis) the presence of the desired EcoRI-HindIII fragment. The single-stranded DNA was also isolated and used for site-directed mutagenesis, carried out as described by Nakamaya et al., 14 Nucl. Acids Res. 9679, 1986. The synthetic oligodeoxynucleotides used for the site-directed mutagenesis were:

1) 5′ AGTTGTGCCTTTGAGAC

2) 5′ GACTTTGCGACAACTAC

3) 5′ CTCAAATTTGCCGGAGC

The recombinant clones containing point mutation were selected by hybridization to the corresponding mutagenic oligonucleotides 5′ [^{32}P]-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. Single-stranded DNA was isolated from the selected clones and the sequence of the complete DNA polymerase gene was determined to check that each clone contained only the desired mutation. The EcoRI-BstBI fragment from the different clones was ligated with T4 DNA ligase to the same sites of plasmid pABw2, which

contains the EcoRI HindIII fragment of plasmid pAZw200 cloned at the corresponding sites of plasmid pT7-3 of the pT7 series (Tabor et al. 82 Proc. Natl. Acad. Sci. U.S.A., 1074, 1985), under the control of the ϕ10 promoter of bacteriophage T7. This EcoRI-BstBI fragment replaces the wild-type sequence in that region by the corresponding mutant sequence. In this way, the recombinant plasmids pABn2D12A, pABn2E14A, pABn2D66A, pABn2D12AD66A and pABn2E14AD66A were selected, containing the corresponding amino acid changes from the amino terminal end of the ϕ29 DNA polymerase. The recombinant plasmids were used to transform *E. coli* BL21 (DE3) cells containing the bacteriophage T7 RNA polymerase gene in the host DNA under the control of the lac uv5 promoter (Studier et al., 189 J. Mol. Biol. 113, 1986) being, therefore, inducible by IPTG. The ampicillin-resistant bacteria were analyzed for the presence of recombinant plasmids. Expression of the ϕ29 DNA polymerase mutant proteins was obtained by addition of 1 mM IPTG to *E. coli* cells containing the recombinant plasmids, grown at 37° C. and incubated for 1 h at 37° C. Five different mutant proteins were obtained, with the following amino acid changes: 1) alanine at position 12 (with reference to the first methionine in the gene encoding p2) in place of the natural aspartic acid (D12A); 2) alanine at position 14 instead of glutamic acid (E14A); 3) alanine at position 66 instead of aspartic acid (D66A); 4) alanine at positions 12 and 66 instead of aspartic acid (D12A, D66A); and 5) alanine at position 14 and 66 (E14A, D66A). The different mutant proteins were purified and their 3'-5' exonuclease activity determined by the above standard assay to be 100–1000 fold lower than that of the wild-type naturally occurring ϕ29 DNA polymerase.

Deposits

Strains pAZW200 (wild type p2 gene), pKC30A1 (wild type p3 gene), pABN2D12AD66A (exonuclease deficient p2 gene having alanine at positions 12 and 66) have been deposited on Mar. 24, 1989, with the ATCC (American type Culture Collect 12301 Parklawn Drive, Rock, Md. 20852 and assigned numbers 67918, 67919, 67920 respectively.

Applicants' and their assignees, Spanish Research Council (Conse]o Superior De Investigaciones Scientificas, Serrano No. 117, 28006, Madrid, SPAIN), acknowledge their responsibility to replace these cultures should they die before the end of the term of a patent issued hereon, 5 years after the last request for a culture, or 30 years, whichever is the longer, and its responsibility to notify the depository of the issuance of such a patent, at which time the deposits will be made irrevocably available to the public. Until that time the deposits will be made available to the Commissioner of Patents under the terms of 37 CFR Section 1-14 and 35 USC Section 112.

Referring to the FIGURE, the oligonucleotides used to form the above mutants were selected by taking into account the amino acid sequence homology with other polymerases and those mutations known to reduce exonuclease activity of DNA polymerase I. Derbyshire et al. 240 Science 199, 1988. Other mutations which are likely to produce suitable exonuclease mutants are shown in the black boxes. Generally, the amino acid at these portions is either deleted or replaced with a different amino acid. Large deletions or multiple replacement of amino acids are also useful in this invention. After mutagenesis, the level of exonuclease activity is measured and the amount of DNA polymerase activity determined to ensure it is sufficient for use in this invention (e.g., for DNA sequencing), being processive and having strand displacement activity.

Uses

DNA polymerases of this invention are useful in the following methods:

Filling in the 3' recessed termini created by digestion of DNA with restriction enzymes; labelling the termini of DNA fragments with protruding 5' ends (filling in reaction); labelling the termini of blunt ended DNA fragments or DNA fragments with protruding 3' termini (exchange reaction); removing the 3' protruding termini of DNA fragments; labelling DNA fragments for use as hybridization probes by partial digestion of double-stranded DNA using the naturally associated 3'-5' exonuclease activity, followed by a filling reaction with labelled dNTPs (displacement reaction); synthesis of long (over 5 10kb) as well as short single-stranded DNA probes containing multiple copies of a desired sequence, obtained by strand-displacement synthesis on single stranded DNA, such long probes may be labelled with labelled dNTPs at a high specific activity; random labelling of double-stranded DNA at a high specific activity by using degenerated oligonucleotide primers; second-strand cDNA synthesis in cDNA cloning; sequencing DNA using a Sanger-type dideoxy system (Sanger et al. 74 Proc. Natl. Acad. Sci. U.S.A. 5463, 1977) on single- and double-stranded DNA templates; sequencing DNA by a plus/minus-type method (Sanger et al., 94 J Mol. Biol. 441, 1975); random mutagenesis of single- and double-stranded DNA templates by using an exonuclease-deficient DNA polymerase under conditions of low DNA replication fidelity; site-directed mutagenesis on double stranded DNA templates; gene amplification or synthesis of long double-stranded DNA fragments using synthetic oligonucleotides as primers; and amplification or synthesis of double stranded DNA fragments using a ϕ29-type DNA replication system including a ϕ29-type DNA polymerase, a terminal protein, any accessory proteins necessary to enhance the reaction, and a ϕ29 type DNA-protein p3 template.

The ϕ29 type DNA polymerases are particularly useful for DNA sequencing, performing a polymerase chain reaction, and for amplification without the need for temperature cycling to produce extremely long strands of DNA. These methods will now be discussed in detail.

EXAMPLE 1: P.C.R.

There follows an example of a polymerase chain reaction using ϕ29 DNA polymerase. In general, the DNA polymerase may simply be used in place of Klenow or Tag polymerases.

0.1 pmol of target DNA are mixed with 300 pmol each of selected oligonucleotides (15–20 mers), and 75 nmol of each deoxynucleoside triphosphate (1N5 mM) in 50 μl of a buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM magnesium chloride. The solution is brought to 95° C. for 10 minutes, and cooled to 30° C. for 1 min in a waterbath. 1 μl containing 20 ng of ϕ29 DNA polymerase (either wild type or an exonuclease mutant) is added to the mixture and the reaction allowed to proceed for 5 min at 30° C., after which the cycle of heating, cooling, adding enzyme, and reacting

is repeated about nine times. The polymerase used is purified by standard procedures.

Prior polymerases used in polymerase chain reactions failed to provide DNA fragments in the size range greater than about 2 kilobases (Saiki et al., 239 Science 487, 1988; Keohavong et al., 71 Gene 211, 1988). This relative short size is probably due to the secondary structure and hinderance produced by reannealing of the DNA fragment. which impedes the progress of these DNA polymerases. Because ϕ29 DNA polymerase has a high processivity and strand displacement ability, it is an ideal enzyme for DNA amplification to produce long amplified molecules.

EXAMPLE 2: DNA SEQUENCING

For DNA sequencing, the sequence procedure using single-stranded DNA as a template was essentially as described by Tabor et al., 84 Proc. Natl. Acad. Sci. U.S.A. 4767, 1987 with some modification.

In the annealing reaction, the annealing reaction mixture (20 μl) contained 2.5 μg of template DNA, 60 ng of primer (a 10-fold molar ratio to the template) in a buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 75 mM NaCl. The mixture was heated to 65° C. for 15 min and then cooled to room temperature (20°-25° C.) over 30 min.

In the labelling reaction, a single labelling reaction was used for all four termination reactions. To the annealing mixture (20 μl) was added 20 μl of a mixture containing 0.6 μM each dGTP, dTTP, dCTP and [α-^{32}P]dATP, 2 mM dithiothreitol, 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 8% glycerol. Labelling was started by addition of the ϕ29 DNA polymerase (either wild type or exonuclease deficient, 150 ng). Incubation was at room temperature for 5 min, at which time the reaction was complete. Four aliquots (8 μl each) of the "labelling" reaction mixture were used for the "termination" reactions.

In the extension-termination reaction, four separate dideoxy "termination" mixtures were prepared in 1.0 ml microcentrifuge tubes. Each mixture (2 μl) contained 20 μM each of the three dNTPs, the remaining dNTP, and its corresponding dideoxy- NTP, being at 2 μM and 200 μM, respectively. Eight microliters of the above labelling reaction mixture was added to each termination mixture and incubated for 15 min at 30° C. Three microliters of stop solution (95% formamide/20 mM EDTA/0.05% xylene cyanol/0.05% bromophenol blue) was then added. The mixtures were heated at 95° C. for 2 min immediately prior to loading 6 μl onto a sequencing gel.

A protocol for double-stranded DNA sequence is similar to the above protocol but preceded by an alkali denaturation step.

For the reasons discussed above, the secondary structure of the DNA template may impede the progress of DNA polymerases. This may occur either at an pallindromic sequence, which may form a hairpin structure, or at other sequences where an enzyme pauses dependent upon a specific sequence. Because of the high processivity and strand displacement ability of ϕ29 DNA polymerases, the sequencing results obtained with this polymerase are superior to those of the prior art.

In the following methods it is useful to include accessory proteins, such as p5 and p6 in the reaction mix. Preparation of p6 is described by Blanco et al., 62 J. Virol. 4167, 1988. Preparation of p5 was as follows:

E. coli K12ΔH1Δtrp cells carrying the gene 5-containing recombinant plasmid pGM26 or *B. subtilis* cells infected with the phage ϕ29 mutant sus 14(1242) were used as a source of protein p5 for purification. The protein p5 present in the *E. coli* extracts amounted to ~1.4% of the total protein after 2.5 h of induction at 42° C., and that present in the *B. subtilis* extracts was ~2.7% of the total protein.

Ten g of *E. coli* K12ΔH1Δtrp cells harboring the gene 5-containing recombinant plasmid pGM26, were induced for 2.5 h at 42° C., were ground with alumina (20 g) and extracted with buffer A (50 mM Tris HCl, pH 7.5 5% glycerol) containing 0.3M KCl. The lysate was centrifuged for 10 min at 16,500×g and the pellet reextracted with the same buffer. The two supernatants were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was dissolved in buffer A, dialyzed against the same buffer, diluted with buffer A 20% glycerol and passed through a DEAE- cellulose column (2.7 cm×10 cm) equilibrated with buffer A +10 mM NaCl. The column was washed first with buffer A +20% glycerol, then with buffer A, and protein p5 was finally eluted with buffer A +50 mM NaCl. The fractions containing protein p5 were pooled and precipitated with ammonium sulfate to 65% saturation. The pellet was resuspended in 1.2 ml of buffer A +1.4M ammonium sulfate and 50% glycerol. The pellet remaining after centrifugation, containing most of protein p5, was dissolved in buffer A +50% glycerol. Protein p5 was purified by a similar procedure from *B. subtilis* cells infected with the ϕ29 delayed lysis mutant sus14(1242). In all purification steps protein p5 was followed by SDS-polyacrylamide gel electrophoresis.

In some preparations, after the last purification step, protein p5 was centrifuged for 24 h at 260,000×g at 0° C. in a 5 ml 15 to 30% (v/v) glycerol gradient in 50 mM Tris-HCl, pH 7.5, 0.2M NaCl. After centrifugation, 0.2 ml fractions were collected and the presence of protein p5 was determined.

EXAMPLE 3: REPLICATIVE-TYPE AMPLIFICATION

As described above, long strands of DNA may be synthesized by primer elongation using a ϕ29-type DNA polymerase. This property may be used to amplify DNA without the need for the temperature cycling used in prior polymerase chain reactions. This process makes use of a protein primer rather than an oligonucleotide primer. Generally, the terminal repeat sequences of a ϕ29-type DNA polymerase are covalently bonded by any of a number of standard methods, to each end of the DNA molecule to be amplified. This bonding may be by direct ligation of the sequence to be amplified, or may be by a procedure similar to site-directed mutagenesis, where an oligonucleotide comprising the terminal sequence is constructed to allow recombination of that nucleotide sequence to adjacent DNA which is required to be amplified. In an alternative method, a restriction endonuclease may be used to randomly cut genomic DNA and synthetic oligonucleotides, comprising the terminal sequences, placed at these sites. In each case the DNA is amplified by provision of p2 and p3 proteins, along with nucleoside triphosphates. An example of this now follows:

A preparation of ϕ29 DNA protein p3 isolated by standard procedure is cut with the restriction nuclease ClaI to produce two fragments of length 6147 and 13138 bp. A DNA fragment containing an appropriate

multicloning site is then ligated to join the two ClaI fragments The DNA fragment to be amplified is then ligated to one of the multicloning sites and the resulting DNA is used as a template for DNA synthesis.

The incubation mixture contains, in 25 μl, 50 mM Tris HCl. pH 7.5. 10 mM $MgCl_2$, 1 mM dithiothreitol. 1 mM spermidine, 20 mM ammonium sulfate, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP, 80 ng of ϕ29 DNA polymerase, 20 ng of terminal protein p3 purified as described (Prieto et al. 81 Proc. Natl. Acad. Sci. U.S.A., 1639, 1984) from *E. coli* N99λts cells harboring the gene 3 containing recombinant plasmid pKC30A1 (Garcia et al., 21 Gene 65, 1983), proteins p5 (9 μg) and p6 (2 μg) purified from ϕ29-infected *B. subtilis* as described above, and the desired amount of template (10 ng-1 μg). After incubation for 60 min. at 30° C., 25 μl containing all the components of the system except the template are added and the mixture is incubated again for 60 min. at 30° C. (2nd cycle). The cycles are repeated in the same way several times.

EXAMPLE 4: SYNTHESIS OF LONG STRANDS OF DNA

The DNA polymerases of this invention permit ready synthesis of very long DNA molecules useful in a large number of applications, e.g., RFLP analysis, and probe construction. There follows an example of this methodology.

Single-stranded M13 DNA was hybridized with a 17-mer M13 oligonucleotide primer. The incubation mixture contained, in 10 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.5 μg of primed M13 DNA, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P] dATP and ϕ29 DNA polymerase (50 ng). After incubation for 40 min at 30° C. the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% sodium dodecyl sulfate and the Cerenkov radiation of the excluded fraction was counted. To analyze the size of the DNA synthesized, a sample was subjected to electrophoresis in alkaline 0.7% agarose gels along with DNA length markers. The DNA markers were detected with ethidium bromide and the synthesized DNA was detected by autoradiography of the dried gel. In 40 min of incubation at 30° C., DNA longer than 70 Kb was synthesized.

Other embodiments are within the following claims.

We claim:

1. A non-naturally occurring DNA fragment consisting essentially of DNA encoding a modified ϕ29-type DNA polymerase, wherein said polymerase comprises sufficient DNA polymerase activity for use in DNA sequencing, and an exonuclease activity which is less than 10% the activity of the corresponding naturally occurring ϕ29-type DNA polymerase.

2. The fragment of claim 1 wherein said DNA fragment encodes a DNA polymerase having substantially no exonuclease activity.

3. The DNA fragment of claim 1 wherein said DNA fragment comprises a DNA sequence encoding a ϕ29 DNA polymerase in which the amino acid moiety at position 12, 14, or 66 of the polymerase is replaced by an alanine moiety.

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United States Patent [19]

Döbeli et al.

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[45] **Date of Patent: Oct. 31, 1989**

[54] **METAL CHELATE RESINS**

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[58] **Field of Search** 525/54.1, 54.3, 54.31, 525/54.32, 54.4, 54.45

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[57] **ABSTRACT**

Metal chelate resins whose complexed nitrilotriacetic acid residues are bound to a carrier matrix via a spacer and which are suitable for metal chelate chromatography of proteins, especially those which contain neighboring histidines.

6 Claims, No Drawings

METAL CHELATE RESINS

Metal chelate affinity chromatography, a new purification method for proteins, was introduced in 1975 by Porath et al. [Nature 258, 598–599 (1975)]. This new technology has meanwhile been used successfully in many places and has already been discussed in review articles [Lëonnerdal, B. and Keen C. L., J. Appl. Biochem. 4, 203–208 (1982); Sulkowski, E., Trends in Biotechnology 3, 1–7 (1985)]. Metal chelate affinity chromatography is based on the discovery that metal ions such as Cu^{2+} and Zn^{2+} bound (immobilized) to a chromatography gel by chelate bonding can take part in a reversible interaction with electron donor groups situated on the surface of proteins, especially the imidazole side-chain of histidine. At a pH value at which the electron donor group is present at least partially in non-protonized form the protein is bonded to the chromatography gel (e.g. agarose) and can subsequently be eluted by means of a buffer with a lower pH value at which the electron donor group is protonized. Iminodiacetic acid, which is bound to the carrier matrix of the resin via a so-called spacer, has, for example, been very reliable as the chelate former.

An ideal chelate resin for the purification of biopolymers must therefore on the one hand strongly complex the metal ions and on the other hand must permit reversible interactions between metal ions and proteins. Immobilized iminodiacetic acid largely fulfils these requirements for Cu^{II} ions, but only to a limited extent for Ni^{II} ions, since the latter are only weakly bonded and are often washed-out even upon loading with the protein mixture. On the other hand, Ni^{II} chelate resins are of particular interest for the purification of biological material, as Ni^{2+} has a high coordination number: Ni^{II} ions complex six ligands, Cu^{II} ions preferably complex four. In nickel complexes four valencies are available for anchoring the metal ions in the resin and two valencies are available for the interchanges between metal ions and biopolymers.

Hitherto there has not been a lack of attempts to manufacture chelate resins with a possible greater affinity to a metal ion. As complex forming components there have been used e.g. N,N,N′-ethylenediaminetriacetic acid [Haner, M. et al., anal. Biochem. 138, 229–234 (1984)] and 1,3-diaminopropane N,N,N′,N′-tetraacetic acid [Moyers, E. M. and J. S. Fritz, Anal. Chem. 49, 418–423 (1977)]. However these resins have the disadvantage that the interchanges between metal ions and biopolymers are not optimal.

SUMMARY OF THE INVENTION

The present invention is concerned with novel resins, which are suitable for metal chelate chromatography, and their manufacture as well as the use of these metal chelate resins for the purification of proteins, especially those which contain neighbouring histidine residues.

DETAILED DESCRIPTION

Nitrilotriacetic acid is a four-pronged chelate former. Immmobilized nitrilotriacetic acid would be a suitable chelate resin for metal ions with the coordination number six, since two valencies are available for the reversible bonding of the biopolymers. Such a metal chelate resin should be especially suitable for the binding of proteins with two neighbouring histidines on its surface.

Nitrilotriacetic acid can, however, not be bound to a carrier analogously to iminodiacetic acid without substantially diminishing its capability of chelate formation. This problem can be solved by the manufacture of novel nitrilotriacetic acid derivatives of the formula

$$NH_2—(CH_2)_x—CH(COOH)—N(CH_2COOH)_2 \quad I$$

wherein x signifies 2, 3 or 4, and their immobilization on a carrier matrix via a spacer.

The present invention is therefore concerned with nitrilotriacetic acid derivatives of the previously mentioned formula and their salts as well as a process for their manufacture. Especially preferred nitrilotriacetic acid derivatives in accordance with the invention are N-[3-amino-1-carboxypropyl]-iminodiacetic acid and N-[5-amino-1-carboxypentyl]-iminodiacetic acid.

The present invention is also concerned with metal chelate resins which are suitable, on the basis of their metal chelate groups, for the purification of proteins, especially those which contain neighbouring histidines, as well as a process for their manufacture.

The metal chelate resins in accordance with the invention are defined by the general formula Carrier matrix-spacer-NH—$(CH_2)_x$—CH(COOH)—$N(CH_2COO^-)_2$ Ni^{2+}, wherein x signifies 2, 3 or 4.

As the carrier matrix there come into consideration materials which are used in affinity and gel chromatography, for example cross-linked dextrans, agarose (especially in the form known under the trade names Sepharose ®, Pharmacia, Uppsala, Sweden) or polyacrylamides.

As the spacer there come into consideration the spacer groups already known from affinity chromatography, with the groups —O—CH_2—CH(OH)—CH_2— and —O—CO— being preferred.

Especially preferred chelate resins in accordance with the invention are those of the formulae

[Agarose or Sepharose ® CL 6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COO^-)_2Ni^{2+}$

and

Agarose—O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COO^-)_2Ni^{2+}$.

The manufacture of the nitrilotriacetic acid derivatives in accordance with the invention can be effected in a manner known per se by reacting a N-terminal protected compound of the formula R—HN—$(CH_2)_x$—$CH(NH_2)$—COOH, wherein R signifies an amino protecting group and x signifies 2, 3 or 4, with bromoacetic acid in an alkaline medium and subsequently cleaving off the protecting group. A preferred amino protecting group is the benzyloxycarbonyl residue (Z), which can be removed by catalytic hydrogenation, preferably with Pd/C. In this manner N^{γ}-Z-L-2,4-diaminobutyric acid and N^{ϵ}-Z-L-lysican be converted into the previously mentioned especially preferred nitrilotriacetic acid derivatives.

The manufacture of the chelate resins in accordance with the invention can be effected in a manner known per se, whereby firstly the carrier matrix is functionalized (introduction of the spacer) and then the desired nitrilotriacetic acid derivative is covalently bonded to the spacer.

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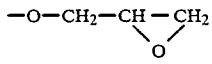
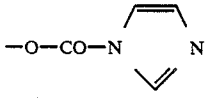
When agarose is used as the carrier matrix it is reacted, for example, with epibromohydrin in an alkaline medium so that there is obtained oxirane-agarose which contains



groups. The oxirane-agarose can then be converted into the desired chelate resin in accordance with the invention in a manner known per se by reaction with a nitriloacetic acid derivative in accordance with the invention, preferably with N-[3-amino-1-carboxypropyl]-iminodiacetic acid or N-[5-amino-1-carboxypentyl]-iminodiacetic acid, in an alkaline medium and subsequent washing with a nickel salt solution, for example with nickel sulphate. In special cases the use of a different metal ion (e.g. Co, Cd) is advantageous, so the corresponding metal chelate can be obtained readily by reacting the resin with a suitable metal salt. Epichlorohydrin can also be used in place of epibromohydrin. As the agarose there is conveniently used a standardized product, preferably Sepharose ® from the firm Pharmacia, Uppsala, Sweden. Sepharose ® Cl-6B is especially suitable. In an analogous manner, polyacrylamide resins which contain free hydroxy groups can be converted into chelate resins in accordance with the invention as previously indicated. When cation exchange resins are used as the matrix, the coupling of the nitrilotriacetic acid derivative can be effected directly with the formation of an amide bond.

For the manufacture of the chelate resins in accordance with the invention there can also be used commercially available, already functionalized carrier matrices. An especially preferred functionalized carrier matrix in connection with the present invention is imidazolecarbamate-agarose which contains



groups and which is marketed under the trade mark Reactigel TM of the firm Pierce, Rockford, IL, USA.

It has been shown that the chelate resins in accordance with the invention are distinguished by an especially high specificity towards peptides and proteins which contain neighbouring histidine residues and are therefore especially suitable for the purification of proteins with neighbouring histidine residues, especially those which contain 2 neighbouring histidine residues. The term "neighbouring histidine residues" refers to the arrangement of the histidine residues of the particular peptides and proteins in three dimensional space, i.e. on the surface of the compounds. The neighbourhood of the histidine residues can be given already on the basis of the primary structure or can be realized only by the secondary and/or tertiary structure. The chelate resins in accordance with the invention are accordingly suitable for the purification of native and denatured proteins which contain several, especially neighbouring, preferably immediately neighbouring, histidine residues.

The chelate resins in accordance with the invention can be used batch-wise or continuously in operating columns. Prior to the loading with protein the chelate resins in accordance with the invention are conveniently equilibrated with an aqueous buffer which itself does not form chelates with nickel, preferably a phosphate buffer, pH 8. The equilibrating buffer (as well as the elution buffer) can contain a denaturing agent or a detergent, for example guanidine.HCl, urea or Triton. The addition of such a denaturing agent or detergent permits problem-free operations even with proteins which are extremely difficultly soluble in aqueous solution such as, for example, membrane proteins. The elution of the protein can be carried out at a constant pH value or with linear or discontinuously falling pH gradients. The optimal elution conditions depend on the amount and type of impurities present, the amount of material to be purified, the column dimensions etc. and are conveniently determined on a case by case basis.

The following Examples illustrate the manufacture of nitrilotriacetic acid derivatives in accordance with the invention as well as the manufacture of metal chelate resins in accordance with the invention and their use in the purification of proteins with neighbouring histidine residues.

EXAMPLE 1

41.7 g of bromoacetic acid were dissolved in 150 ml of 2N sodium hydroxide solution and cooled to 0° C. Thereto there was slowly added dropwise at 0° C. while stirring a solution of 42 g of N^{ϵ}-Z-L-lysine in 225 ml of 2N sodium hydroxide solution. After 2 hours the cooling was removed and the mixture was stirred overnight. The reaction mixture was then held at 50° C. for 2 hours and 540 ml of 1N hydrochloric acid were subsequently added. After the mixture had been cooled the separated crystals were filtered off. The product was dissolved in 1N sodium hydroxide solution and again precipitated with the same amount of 1N hydrochloric acid and filtered off. There were obtained 40 g of N-[5-benzyloxycarbonylamino-1-carboxypentyl]-iminodiacetic acid in the form of white crystals, m.p. 172°-174° C. (dec.), $[\alpha]_D = +9.9°$ (c=1; 0.1N NaOH).

7.9 g of the lysine derivative obtained were dissolved in 49 ml of 1N sodium hydroxide solution and, after the addition of a spatula tip of 5% Pd/C, hydrogenated at room temperature and normal pressure. The catalyst was filtered off and the filtrate was evaporated. There resulted 6.2 g of N-[5-amino-1-carboxypentyl]-iminodiacetic acid whose structure, $NH_2—(CH_2)_4—CH(COOH)—N—(CH_2COOH)_2$— was confirmed by the NMR spectrum.

100 ml of Sepharose ® CL-6B (Pharmacia) were washed twice on a glass suction filter with about 500 ml of water and then reacted at 30° C. for 4 hours in a 500 ml round flask with 16 ml of 4N sodium hydroxide solution and 8.22 ml of epibromohydrin. The total volume of the reaction mixture was 200 ml. The activated Sepharose was subsequently filtered off, washed neutral with water and transferred back into the reaction vessel. 6.5 g of N-[5-amino-1-carboxypentyl]-iminodiacetic acid were dissolved in 50 ml of water and added to the activated Sepharose together with 10.6 g of solid sodium carbonate. The mixture was stirred slowly at 60° C. overnight. The resulting chelate resin with the formula [Sepharose ® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—N($CH_2COOH)_2$ (NTA resin) was subsequently washed in a chromatography column in succession with 500 ml of water, 100 ml of aqueous $NiSO_4 \cdot 6H_2O$ (2 wt.%), 200 ml

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of water, 200 ml of 0.2M acetic acid (containing 0.2M NaCl and 0.1 wt./vol.% Tween 20) and 200 ml of water. The nickel ion concentration in the resulting chelate resin of the formula [Sepharose ® CL-6B]—O—CH-$_2$—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH-)—$N(CH_2COO^-)_2Ni^{2+}$ amounted to about 7.1 micromol/ml.

EXAMPLE 2

For a qualitative comparison of the stabilities of the nickel complexes of immobilized iminodiacetic acid (IMA) and imobilized nitrilotriacetic acid (NTA), the two nickel chelate resins were eluted with an aqueous solution of iminodiacetic acid and the washing out of the nickel ions was followed.

50 ml of IMA resin of the formula Agarose—O—CH_2—CH(OH)—CH_2—$N(CH_2COOH)_2$ (preparation see European Patent Application No. 84101814.6, Publication No. 118 808) were placed in a chromatography column (d=1.6 cm) and washed well with water. Then, 10 ml of a 0.012M $NiSO_4 \cdot 5H_2O$ solution in water were introduced at a flow rate of 100 ml/h and the column was subsequently washed with 70 ml of water. It was eluted with 0.1M aqueous iminodiacetic acid (IMA), pH 7.0. 10 ml fractions were collected. Nickel ions could be detected (UV 390 nm) in fractions 10–19.

In the same manner, 50 ml of NTA resin of the structure [Sepharose ® CL-6B]—O—CH_2—CH(OH)—CH-$_2$—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COOH)_2$ *were placed in a chromatography column* (*d*=1.6 cm), washed with water, thereafter loaded with 10 ml of 0.012M $NiSO_4 \cdot 5H_2O$, again washed with water and eluted with 0.1M aqueous iminodiacetic acid, pH 7.0. Nickel ions could only be detected (UV 390 nm) in fractions 30–34, from which it is evident that the Ni^{II} ions are bound more strongly in the novel NTA resin than in the known IMA resin.

EXAMPLE 3

6.5 g of bromoacetic acid were dissolved in 8.1 ml of 4N sodium hydroxide solution and cooled to 0° C. Thereto there was added dropwise while stirring a solution of 4.9 g of N^{γ}-benzyloxycarbonyl-L-2,4-diaminobutyric acid in 24.4 ml of 2N sodium hydroxide solution. After 2 hours the cooling was removed and the mixture was stirred overnight. The reaction mixture was then held at 50° C. for 2 hours and 12.2. ml of 4N hydrochloric acid were subsequently added. After the mixture had been cooled the separated crystals were filtered off. The product was dissolved in 2N sodium hydroxide solution and again precipitated with 6.1 ml of 4N hydrochloric acid and filtered off. There were obtained 5 g of N-[3-benzyloxycarbonylamino-1-carboxypropyl]-iminodiacetic acid in the form of white crystals, m.p. 136°–138° C. (dec.).

2.9 g of the butyric acid derivative obtained were dissolved in 16 ml of 1N sodium hydroxide solution and, after the addition of a spatula tip of 5% Pd/C, hydrogenated at room temperature and normal pressure. The catalyst was filtered off and the filtrate was evaporated. There resulted 2.2 g of N-[3-amino-1-carboxypropyl]-iminodiacetic acid whose structure, NH_2—$(CH_2)_2$—CH(COOH)—$N(CH_2COOH)_2$, was confirmed by the NMR spectrum.

A solution of 1.9 g of the N-[3-amino-1-carboxypropyl]-iminodiacetic acid obtained in 50 ml of water was treated with 2.6 g of solid sodium carbonate. To the mixture, cooled to 0° C., were added 50 ml of agarose activated with imidazolecarbamate (Reacti-Gel TM of the firm Pierce). After incubation at 0° C. for 15 hours the resulting chelate resin of the formula Aragrose—O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COOH)_2$ was filtered off, washed with water and loaded with Ni^{II} ions as described in Example 1. The nickel ion concentration in the resulting chelate resin of the formula Agarose—O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COO^-)Ni^{2+}$ amounted to 3.1 micromol/ml.

EXAMPLE 4

A column (ϕ 1 cm, length=4.8 cm) was filled with metal-free chelate resin of the formula [Sepharose ® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COOH)_2$ (NTA resin) and the resin was brought into the nickel form by rinsing with a three-fold column volume of 0.1M $NiSO_4 \cdot 5H_2O$ and subsequently washing with a three-fold columnn volume of 0.2M acetic acid. It was subsequently equilibrated with 0.1M sodium phosphate buffer (pH 8.0) and 0.5 NaCl (flow in each case 13.2 ml/hr.).

1 mg of a model peptide of the formula His-His-Leu-Gly-Gly-Ala-Lys-Glu-Ala-Gly-Asp-Val was taken up in 1 ml of equilibration buffer and applied on to the column. The model peptide could be eluted by washing with 0.2M imidazole in 0.1M sodium phosphate, pH 8.0, and 0.5M NaCl. The detection in the eluate was effected with ninhydrin according Moore, S. and Stein, W. [J. Biol. Chem. 176, 367–388 (1948)].

EXAMPLE 5

In a manner analogous to Example 4, a column (ϕ=1 cm, length=4.8 cm) was filled with NTA resin and the resin was brought into the nickel form. After washing with 0.2M acetic acid the column was equilibrated with 7M guanidine·HCl in 0.1M sodium phosphate buffer (pH 8.0).

Different amounts (up to 12.7 mg) of a model peptide with the formula Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Ser were dissolved in 1 ml of 7M guanidine·HCl and 0.1M sodium phosphate (pH 8.0) and applied on to the column. This peptide is very well soluble in 7M guanidine·HCl, but is poorly soluble in 0.1M sodium phosphate and 0.5M NaCl. The elution was effected by lowering the pH value stepwise. The peptide was detected by means of UV spectrometry at λ=280 nm.

Trypsin from bovine pancreas and cytochrome C from horse heart were used as comparative substances. Neither of the two proteins bonded to the NTA resin at pH 8. Obviously the arrangement of the histidines plays a decisive role. In the case of trypsin three histidines are situated in positions 29, 46 and 79, which in spite of the breaking of the stucture by 7M guanidine are not in the position to form a stable complex and in the case of cytochem C the two histidines are indeed spacially neighbouring (positions 18 and 26), but are not in the position to form a two-pronged ligand, since one histidine is bonded to the haem-iron.

EXAMPLE 6

Lactate dehydrogenase isoenzymes are tetrameric proteins with a molecular weight of 140,000. The isoenzymes from hogs are largely homologous with the exception of the amino terminal region. This is situated on the protein surface. The heart type isoenzyme has no

histidine in this region, but the muscle type has three, among them the sequence His-Val-Pro-His [L. Li et al., J. Biol. Chem. 258, 7029–7032 (1983)].

As described in Example 4, a column (ϕ=1 cm, length=4.8 cm) was filled with NTA resin, the resin was brought into the nickel form and equilibrated with 0.1M sodium phosphate buffer (pH 7.5) and 0.5M NaCl. 2 mg of lactate dehydrogenase from hog heart (H_4-LOH) or hog muscle (M_4-LOH) were taken up in 1.5 ml of equilibration buffer and applied to the column. While H_4-LOH was not adsorbed in spite of its 28 histidine residues, M_4-LOH was adsorbed at pH 7.5 and could be eluted by lowering the pH value to 6.

This experiment shows that the NTA resin is extremely selective for proteins which have as a structural element neighbouring histidines on the protein surface.

We claim:

1. A metal chelate resin of the formula:

carrier matrix-spacer—NH—$(CH_2)_x$—CH(COOH-)—N$(CH_2COO^-)_2Ni^{2+}$

wherein X=2–4.

2. The metal chelate resin of claim **1** wherein the carrier matrix is cross-linked agarose.

3. The metal chelate resin of claim **2** wherein the spacer is —O—CO—.

4. A method for the manufacture of a metal chelate resin comprising the steps of:

a) reacting a carrier matrix with a spacer;

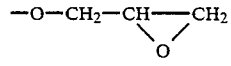
b) reacting the carrier matrix-spacer complex with a compound of the formula:

H_2N—$(CH_2)_X$—CH(COOH)—N$(CH_2COOH)_2$

wherein X=2–4; and

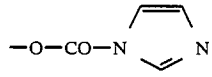
c) washing with a nickel salt solution.

5. The method of claim **8**, wherein the carrier matrix-spacer complex is oxirane-agarose which contains



groups.

6. The method of claim **8** wherein the fuctionalized carrier matrix-spacer complex is imidazolecarbamate-agarose which contains



groups.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 4,877,830

DATED : October 31, 1989

INVENTOR(S) : Heinz Dobeli & Erich Hochuli

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 8, Claim 5, line 11, delete "Claim 8" and insert --Claim 4--.

Column 8, Claim 6, line 19, delete "Claim 8" and insert --Claim 4--.

Signed and Sealed this

Sixth Day of November, 1990

Attest:

Attesting Officer

HARRY F. MANBECK, JR.

Commissioner of Patents and Trademarks

United States Patent [19]

Döbeli et al.

[11] **Patent Number: 5,047,513**

[45] **Date of Patent: Sep. 10, 1991**

[54] **METAL CHELATE RESINS**

[75] Inventors: **Heinz Döbeli**, Ziefen; **Frich Hochuli**, Arisdorf, both of Switzerland

[73] Assignee: **Hoffmann-La Roche Inc.**, Nutley, N.J.

[21] Appl. No.: **596,634**

[22] Filed: **Oct. 15, 1990**

Related U.S. Application Data

[60] Continuation of Ser. No. 396,718, Aug. 22, 1989, abandoned, which is a division of Ser. No. 72,452, Jul. 14, 1987, Pat. No. 4,877,830.

[51] **Int. Cl.**[5] **A23J 1/00;** C07K 3/00; C07K 13/00; C07K 15/00

[52] **U.S. Cl.** **530/412;** 530/417; 210/656; 515/54.1; 515/54.3; 515/54.31; 515/54.32; 515/54.4; 515/54.45

[58] **Field of Search** 530/412, 417; 210/656; 525/54.1, 54.3, 54.31, 54.32, 54.4, 54.45

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,569,794 2/1986 Smith et al. 530/344

OTHER PUBLICATIONS

Lönnerdal et al., "Metal Chelate Affinity Chromatography of Proteins", J. of App. Biochem. 4, 203–208 (1982), pp. 203–208.

Primary Examiner—Nathan M. Nutter
Attorney, Agent, or Firm—George M. Gould; William H. Epstein; Dennis P. Tramaloni

[57] **ABSTRACT**

Metal chelate resins whose complexed nitrilotriacetic acid residues are bound to a carrier matrix via a spacer and which are suitable for metal chelate chromatography of proteins, especially those which contain neighboring histidines.

2 Claims, No Drawings

EXHIBIT L

METAL CHELATE RESINS

This application is a continuation of application Ser. No. 07/396,718, filed 8/22/89, now abandoned, which is a division of application Ser. No. 07/72,452 filed July 14, 1987 and now U.S. Pat. No. 4,877,830.

Metal chelate affinity chromatography, a new purification method for proteins, was introduced in 1975 by Porath et al. [Nature 258, 598–599 (1975)]. This new technology has meanwhile been used successfully in many places and has already been discussed in review articles [Lönnerdal, B. and Keen C. L., J. Appl. Biochem. 4, 203–208 (1982); Sulkowski, E., Trends in Biotechnology 3, 1–7 (1985)]. Metal chelate affinity chromatography is based on the discovery that metal ions such as Cu^{2+} and Zn^{2+} bound (immobilized) to a chromatography gel by chelate bonding can take part in a reversible interaction with electron donor groups situated on the surface of proteins, especially the imidazole side-chain of histidine. At a pH value at which the electron donor group is present at least partially in non-protonized form the protein is bonded to the chromatography gel (e.g. agarose) and can subsequently be eluted by means of a buffer with a lower pH value at which the electron donor group is protonized. Iminodiacetic acid, which is bound to the carrier matrix of the resin via a so-called spacer, has, for example, been very reliable as the chelate former.

An ideal chelate resin for the purification of biopolymers must therefore on the one hand strongly complex the metal ions and on the other hand must permit reversible interactions between metal ions and proteins. Immobilized iminodiacetic acid largely fulfils these requirements for Cu^{II} ions, but only to a limited extent for Ni^{II} ions, since the latter are only weakly bonded and are often washed-out even upon loading with the protein mixture. On the other hand, Ni^{II} chelate resins are of particular interest for the purification of biological material, as Ni^{2+} has a high coordination number: Ni^{II} ions complex six ligands, Cu^{II} ions preferably complex four. In nickel complexes four valencies are available for anchoring the metal ions in the resin and two valencies are available for the interchanges between metal ions and biopolymers.

Hitherto there has not been a lack of attempts to manufacture chelate resins with a possible greater affinity to a metal ion. As complex forming components there have been used e.g. N,N,N′-ethylenediaminetriacetic acid [Haner, M. et al., Anal. Biochem. 138, 229–234 (1984)] and 1,3-diaminopropane N,N,N′-tetraacetic acid [Moyers, E. M. and J. S. Fritz, Anal. Chem. 49, 418–423 (1977)]. However, these resins have the disadvantage that the interchanges between metal ions and biopolymers are not optimal.

SUMMARY OF THE INVENTION

The present invention is concerned with novel resins, which are suitable for metal chelate chromatography, and their manufacture as well as the use of these metal chelate resins for the purification of proteins, especially those which contain neighbouring histidine residues.

DETAILED DESCRIPTION

Nitrilotriacetic acid is a four-pronged chelate former. Immobilized nitrilotriacetic acid would be a suitable chelate resin for metal ions with the coordination number six, since two valencies are available for the reversible bonding of the biopolymers. Such a metal chelate resin should be especially suitable for the binding of proteins with two neighbouring histidines on its surface.

Nitrilotriacetic acid can, however, not be bound to a carrier analogously to iminodiacetic acid without substantially diminishing its capability of chelate formation. This problem can be solved by the manufacture of novel nitrilotriacetic acid derivatives of the formula

$$NH_2—(CH_2)_x—CH(COOH)—N(CH_2COOH)_2 \quad I$$

wherein x signifies 2, 3 or 4, and their immobilization on a carrier matrix via a spacer.

The present invention is therefore concerned with nitrilotriacetic acid derivatives of the previously mentioned formula and their salts as well as a process for their manufacture. Especially preferred nitrilotriacetic acid derivatives in accordance with the invention are N-[3-amino-1-carboxypropyl]-iminodiacetic acid and N-[5-amino-1-carboxypentyl]-iminodiacetic acid.

The present invention is also concerned with metal chelate resins which are suitable, on the basis of their metal chelate groups, for the purification of proteins, especially those which contain neighbouring histidines, as well as a process for their manufacture.

The metal chelate resins in accordance with the invention are defined by the general formula Carrier matrix-spacer-NH—$(CH_2)_x$—CH(COOH)—$N(CH_2COO^-)_2$ Ni^{2+}, wherein x signifies 2, 3 or 4.

As the carrier matrix there come into consideration materials which are used in affinity and gel chromatography, for example cross-linked dextrans, agarose (especially in the form known under the trade names Sepharose ®, Pharmacia, Uppsala, Sweden) or polyacrylamides.

As the spacer there come into consideration the spacer groups already known from affinity chromatography, with the groups —O—CH_2—CH(OH)—CH_2— and —O—CO— being preferred.

Especially preferred chelate resins in accordance with the invention are those of the formulae

[Agarose or
Sepharose ® CL 6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—
—CH(COOH)—$N(CH_2COO^-)_2Ni^{2+}$

and

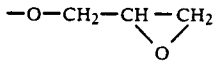
Agarose-O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COO^-)_2$
Ni^{2+}.

The manufacture of the nitrilotriacetic acid derivatives in accordance with the invention can be effected in a manner known per se by reacting a N-terminal protected compound of the formula R—HN—$(CH_2)_x$—$CH(NH_2)$—COOH, wherein R. signifies an amino protecting group and x signifies 2, 3 or 4, with bromoacetic acid in an alkaline medium and subsequently cleaving off the protecting group. A preferred amino protecting group is the benzyloxycarbonyl residue (Z), which can be removed by catalytic hydrogenation, preferably with Pd/C. In this manner N^{γ}-Z-L-2,4-diaminobutyric acid and N^{ϵ}-Z-L-lysine can be converted into the previously mentioned especially preferred nitrilotriacetic acid derivatives.

The manufacture of the chelate resins in accordance with the invention can be effected in a manner known per se, whereby firstly the carrier matrix is functionalized (introduction of the spacer) and then the desired

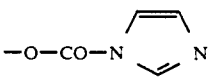
nitrilotriacetic acid derivative is covalently bonded to the spacer.

When agarose is used as the carrier matrix it is reacted, for example, with epibromohydrin in an alkaline medium so that there is obtained oxirane-agarose which contains



groups. The oxirane-agarose can then be converted into the desired chelate resin in accordance with the invention in a manner known per se by reaction with a nitriloacetic acid derivative in accordance with the invention, preferably with N-[3-amino-1-carboxypropyl]-iminodiacetic acid or N-[5-amino-1-carboxypentyl]-iminodiacetic acid, in an alkaline medium and subsequent washing with a nickel salt solution, for example with nickel sulphate. In special cases the use of a different metal ion (e.g. Co, Cd) is advantageous, so the corresponding metal chelate can be obtained readily by reacting the resin with a suitable metal salt. Epichlorohydrin can also be used in place of epibromohydrin. As the agarose there is conveniently used a standardized product, preferably Sepharose ® from the firm Pharmacia, Uppsala, Sweden. Sepharose ® Cl-6B is especially suitable. In an analogous manner, polyacrylamide resins which contain free hydroxy groups can be converted into chelate resins in accordance with the invention as previously indicated. When cation exchange resins are used as the matrix, the coupling of the nitrilotriacetic acid derivative can be effected directly with the formation of an amide bond.

For the manufacture of the chelate resins in accordance with the invention there can also be used commercially available, already functionalized carrier matrices. An especially preferred functionalized carrier matrix in connection with the present invention is imidazolecarbamate-agarose which contains



groups and which is marketed under the trade mark Reactigel TM of the firm Pierce, Rockford, Ill., U.S.A.

It has been shown that the chelate resins in accordance with the invention are distinguished by an especially high specificity towards peptides and proteins which contain neighbouring histidine residues and are therefore especially suitable for the purification of proteins with neighbouring histidine residues, especially those which contain 2 neighbouring histidine residues. The term "neighbouring histidine residues" refers to the arrangement of the histidine residues of the particular peptides and proteins in three dimensional space, i.e. on the surface of the compounds. The neighbourhood of the histidine residues can be given already on the basis of the primary structure or can be realized only by the secondary and/or tertiary structure. The chelate resins in accordance with the invention are accordingly suitable for the purification of native and denatured proteins which contain several, especially neighbouring, preferably immediately neighbouring, histidine residues.

The chelate resins in accordance with the invention can be used batch-wise or continuously in operating columns. Prior to the loading with protein the chelate resins in accordance with the invention are conveniently equilibrated with an aqueous buffer which itself does not form chelates with nickel, preferably a phosphate buffer, pH 8. The equilibrating buffer (as well as the elution buffer) can contain a denaturing agent or a detergent, for example guanidine.HCl, urea or Triton. The addition of such a denaturing agent or detergent permits problem-free operations even with proteins which are extremely difficultly soluble in aqueous solution such as, for example, membrane proteins. The elution of the protein can be carried out at a constant pH value or with linear or discontinuously falling pH gradients. The optimal elution conditions depend on the amount and type of impurities present, the amount of material to be purified, the column dimensions etc. and are conveniently determined on a case by case basis.

The following Examples illustrate the manufacture of nitrilotriacetic acid derivatives in accordance with the invention as well as the manufacture of metal chelate resins in accordance with the invention and their use in the purification of proteins with neighbouring histidine residues.

EXAMPLE 1

41.7 g of bromoacetic acid were dissolved in 150 ml of 2N sodium hydroxide solution and cooled to 0° C. Thereto there was slowly added dropwise at 0° C. while stirring a solution of 42 g of N^{ϵ}-Z-L-lysine in 225 ml of 2N sodium hydroxide solution. After 2 hours the cooling was removed and the mixture was stirred overnight. The reaction mixture was then held at 50° C. for 2 hours and 450 ml of 1N hydrochloric acid were subsequently added. After the mixture had been cooled the separated crystals were filtered off. The product was dissolved in 1N sodium hydroxide solution and again precipitated with the same amount of 1N hydrochloric acid and filtered off. There were obtained 40 g of N-[5-benzyloxycarbonylamino-1-carboxypentyl]-iminodiacetic acid in the form of white crystals, m.p. 172°-174° C. (dec.), $[\alpha]_D = +9.9°$ (c=1; 0.1N NaOH).

7.9 g of the lysine derivative obtained were dissolved in 49 ml of 1N sodium hydroxide solution and, after the addition of a spatula tip of 5% Pd/C, hydrogenated at room temperature and normal pressure. The catalyst was filtered off and the filtrate was evaporated. There resulted 6.2 g of N-[5-amino-1-carboxypentyl]-iminodiacetic acid whose structure, $NH_2—(CH_2)_4—CH(COOH)—N—(CH_2COOH)_2$, was confirmed by the NMR spectrum.

100 ml of Sepharose ® CL-6B (Pharmacia) were washed twice on a glass suction filter with about 500 ml of water and then reacted at 30° C. for 4 hours in a 500 ml round flask with 16 ml of 4N sodium hydroxide solution and 8.22 ml of epibromohydrin. The total volume of the reaction mixture was 200 ml. The activated Sepharose was subsequently filtered off, washed neutral with water and transferred back into the reaction vessel. 6.5 g of N-[5-amino-1-carboxypentyl]-iminodiacetic acid were dissolved in 50 ml of water and added to the activated Sepharose together with 10.6 g of solid sodium carbonate. The mixture was stirred slowly at 60° C. overnight. The resulting chelate resin with the formula [Sepharose ® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—N($CH_2COOH)_2$ (NTA resin) was subsequently washed in a chromatography column in succession with 500 ml of

water, 100 ml of aqueous $NiSO_4.6H_2O$ (2 wt. %), 200 ml of water, 200 ml of 0.2M acetic acid (containing 0.2M NaCl and 0.1 wt./vol. % Tween 20) and 200 ml of water. The nickel ion concentration in the resulting chelate resin of the formula [Sepharose® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COO^-)_2Ni^{2+}$ amounted to about 7.1 micromol/ml.

EXAMPLE 2

For a qualitative comparison of the stabilities of the nickel complexes of immobilized iminodiacetic acid (IMA) and imobilized nitrilotriacetic acid (NTA), the two nickel chelate resins were eluted with an aqueous solution of iminodiacetic acid and the washing out of the nickel ions was followed.

50 ml of IMA resin of the formula Agarose—O—CH_2—CH(OH)—CH_2—$N(CH_2COOH)_2$ (preparation see European Patent Application No. 84101814.6, Publication No. 118 808) were placed in a chromatography column (d = 1.6 cm) and washed well with water. Then, 10 ml of a 0.012M $NiSO_4.5H_2O$ solution in water were introduced at a flow rate of 100 ml/h and the column was subsequently washed with 70 ml of water. It was eluted with 0.1M aqueous iminodiacetic acid (IMA), pH 7.0. 10 ml fractions were collected. Nickel ions could be detected (UV 390 nm) in fractions 10–19.

In the same manner, 50 ml of NTA resin of the structure [Sepharose® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COOH)_2$ were placed in a chromatography column (d = 1.6 cm), washed with water, thereafter loaded with 10 ml of 0.012M $NiSO_4.5H_2O$, again washed with water and eluted with 0.1M aqueous iminodiacetic acid, pH 7.0. Nickel ions could only be detected (UV 390 nm) in fractions 30–34, from which it is evident that the Ni^{II} ions are bound more strongly in the novel NTA resin than in the known IMA resin.

EXAMPLE 3

6.5 g of bromoacetic acid were dissolved in 8.1 ml of 4N sodium hydroxide solution and cooled to 0° C. Thereto there was added dropwise while stirring a solution of 4.1 g of N^{γ}-benzyloxycarbonyl-L-2,4-diaminobutyric acid in 24.4 ml of 2N sodium hydroxide solution. After 2 hours the cooling was removed and the mixture was stirred overnight. The reaction mixture was then held at 50° C. for 2 hours and 12.2 ml of 4N hydrochloric acid were subsequently added. After the mixture had been cooled the separated crystals were filtered off. The product was dissolved in 2N sodium hydroxide solution and again precipitated with 6.1 ml of 4N hydrochloric acid and filtered off. There were obtained 5 g of N-[3-benzyloxycarbonylamino-1-carboxypropyl]-iminodiacetic acid in the form of white crystals, m.p. 136°–138° C. (dec.).

2.9 g of the butyric acid derivative obtained were dissolved in 16 ml of 1N sodium hydroxide solution and, after the addition of a spatula tip of 5% Pd/C, hydrogenated at room temperature and normal pressure. The catalyst was filtered off and the filtrate was evaporated. There resulted 2.2 g of N-[3-amino-1-carboxypropyl]-iminodiacetic acid whose structure, NH_2—$(CH_2)_2$—CH(COOH)—$N(CH_2COOH)_2$, was confirmed by the NMR spectrum.

A solution of 1.9 g of the N-[3-amino-1-carboxypropyl]-iminodiacetic acid obtained in 50 ml of water was treated with 2.6 g of solid sodium carbonate. To the mixture, cooled to 0° C., were added 50 ml of agarose activated with imidazolecarbamate (Reacti-Gel™ of the firm Pierce). After incubation at 0° C. for 15 hours the resulting chelate resin of the formula Agarose—O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COOH)_2$ was filtered off, washed with water and loaded with Ni^{II} ions as described in Example 1. The nickel ion concentration in the resulting chelate resin of the formula Agarose—O—CO—NH—$(CH_2)_2$—CH(COOH)—$N(CH_2COO^-)Ni^{2+}$ amounted to 3.1 micromol/ml.

EXAMPLE 4

A column (ϕ1 cm, length = 4.8 cm) was filled with metal-free chelate resin of the formula [Sepharose® CL-6B]—O—CH_2—CH(OH)—CH_2—NH—$(CH_2)_4$—CH(COOH)—$N(CH_2COOH)_2$ (NTA resin) and the resin was brought into the nickel form by rinsing with a three-fold column volume of 0.1M $NiSO_4.5H_2O$ and subsequently washing with a three-fold column volume of 0.2M acetic acid. It was subsequently equilibrated with 0.1M sodium phosphate buffer (pH 8.0) and 0.5M NaCl (flow in each case 13.2 ml/hr.).

1 mg of a model peptide of the formula His-His-Leu-Gly-Gly-Ala-Lys-Glu-Ala-Gly-Asp-Val was taken up in 1 ml of equilibration buffer and applied on to the column. The model peptide could be eluted by washing with 0.2M imidazole in 0.1M sodium phosphate, pH 8.0, and 0.5M NaCl. The detection in the eluate was effected with ninhydrin according Moore, S. and Stein, W. [J. Biol. Chem. 176, 367–388 (1948)].

EXAMPLE 5

In a manner analogous to Example 4, a column (ϕ = 1 cm, length = 4.8 cm) was filled with NTA resin and the resin was brought into the nickel form. After washing with 0.2M acetic acid the column was equilibrated with 7M guanidine.HCl in 0.1M sodium phosphate buffer (pH 8.0).

Different amounts (up to 12.7 mg) of a model peptide with the formula Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Ser were dissolved in 1 ml of 7M guanidine.HCl and 0.1M sodium phosphate (pH 8.0) and applied on to the column. This peptide is very well soluble in 7M guanidine.HCl, but is poorly soluble in 0.1M sodium phosphate and 0.5M NaCl. The elution was effected by lowering the pH value stepwise. The peptide was detected by means of UV spectrometry at λ = 280 nm.

Trypsin from bovine pancreas and cytochrome C from horse heart were used as comparative substances. Neither of the two proteins bonded to the NTA resin at pH 8. Obviously the arrangement of the histidines plays a decisive role. In the case of trypsin three histidines are situated in positions 29, 46 and 79, which is spite of the breaking of the stucture by 7M guanidine are not in the position to form a stable complex and in the case of cytochem C the two histidines are indeed specially neighbouring (positions 18 and 26), but are not in the position to form a two-pronged ligand, since one histidine is bonded to the haem-iron.

EXAMPLE 6

Lactate dehydrogenase isoenzymes are tetrameric proteins with a molecular weight of 140,000. The isoenzymes from hogs are largely homologous with the exception of the amino terminal region. This is situated on

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the protein surface. The heart type isoenzyme has no histidine in this region, but the muscle type has three, among them the sequence His-Val-Pro-His [L. Li et al., J. Biol. Chem. 258, 7029–7032 (1983)].

As described in Example 4, a column (ϕ=1 cm, length=4.8 cm) was filled with NTA resin, the resin was brought into the nickel form and equilibrated with 0.1M sodium phosphate buffer (pH 7.5) and 0.5M NaCl. 2 mg of lactate dehydrogenase from hog heart (H_4-LOH) or hog muscle (M_4-LOH) were taken up in 1.5 ml of equilibration buffer and applied to the column. While H_4-LOH was not adsorbed in spite of its 28 histidine residues, M_4-LOH was adsorbed at pH 7.5 and could be eluted by lowering the pH value to 6.

This experiment shows that the NTA resin is extremely selective for proteins which have as a structural element neighbouring histidines on the protein surface.

We claim:

1. A method for the purification of proteins comprising subjecting said proteins to affinity chromatography on a metal chelate resin of the formula:

Carrier
matrix-spacer-NH—$(CH_2)_X$—CH(COOH-)—$N(CH_2COO^-)_2Ni^{2+}$

wherein X=2–4.

2. The method of claim **1** wherein the proteins contain several neighboring histidine residues.

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